Translating complexity and heterogeneity of pancreatic tumor: 3D in vitro to in vivo models

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Translating complexity and heterogeneity of pancreatic tumor: 3D *in vitro* to *in vivo* models ☆

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive type of cancer with an overall survival rate of less than 7–8%, emphasizing the need for novel effective therapeutics against PDAC. However only a fraction of therapeutics which seemed promising in the laboratory environment will eventually reach the clinic. One of the main reasons behind this low success rate is the complex tumor microenvironment (TME) of PDAC, a highly fibrotic and dense stroma surrounding tumor cells, which supports tumor progression as well as increases the resistance against the treatment. In particular, the growing understanding of the PDAC TME points out a different challenge in the development of efficient therapeutics – a lack of biologically relevant *in vitro* and *in vivo* models that resemble the complexity and heterogeneity of PDAC observed in patients. The purpose and scope of this review is to provide an overview of the recent developments in different *in vitro* and *in vivo* models, which aim to recapitulate the complexity of PDAC in a laboratory environment, as well to describe how 3D *in vitro* models can be integrated into drug development pipelines that are already including sophisticated *in vivo* models. Hereby a special focus will be given on the complexity of *in vivo* models and the challenges *in vitro* models face to reach the same levels of complexity in a controllable manner. First, a brief introduction of novel developments in two dimensional (2D) models and *ex vivo* models is provided. Next, recent developments in three dimensional (3D) *in vitro* models are described ranging from spheroids, organoids, scaffold models, bioprinted models to organ-on-chip models including a discussion on advantages and limitations for each model. Furthermore, we will provide a detailed overview on the current PDAC *in vivo* models including chemically-induced models, syngeneic and xenogeneic models, highlighting hetero- and orthotopic, patient-derived tissues (PDX) models, and genetically engineered mouse models. Finally, we will provide a discussion on overall limitations of both, *in vitro* and *in vivo* models, and discuss necessary steps to overcome these limitations to reach an efficient drug development pipeline, as well as discuss possibilities to include novel *in silico* models in the process.

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1. Introduction

Pancreatic cancer is an extremely aggressive and lethal type of cancer with an overall 5-year survival rate of less than 7–8% [1–5]. Around 90–95% of malignancies in the pancreas arise from the exocrine tissue, including pancreatic ductal adenocarcinoma (PDAC) which is the most prevalent and aggressive type of pancreatic cancer accounting for around 70–80% of all pancreatic cancers [1–3]. In particular the lack of specific symptoms renders early diagnosis challenging, often leading to patients receiving treatment at an already advanced stage of the disease. Conventional chemotherapy is still the current standard of care for advanced, non-resectable PDAC, however, they provide only a few months of survival benefit for patients. Despite tremendous efforts in the development of novel therapeutics for the treatment as well as novel techniques for the diagnosis of PDAC, the overall mortality and incidence has increased over recent years and predicted to further increase in the future [1,6].

The growing number of patients emphasizes the urgent need for effective novel therapeutics against PDAC. However, only a fraction of therapeutics that seem successful in the laboratory will eventually reach or pass clinical trials [1]. One of the main reasons for the lack of clinical efficiency of such therapeutics is the tumor microenvironment (TME), which is highly desmoplastic and commonly known as the tumor stroma. The PDAC stroma is highly heterogeneous and the role of different components of the tumor stroma is still under debate. While PDAC progresses, it changes the surrounding stroma towards a tumor-promoting environment, which is a process crucial for tumor growth, metastasis and resistance to applied treatments [1,7]. With increasing understanding of the importance of the stroma in PDAC progression, several treatments have emerged in recent years that particularly focus on the modulation of the stroma than depletion. Examples include inhibiting the function of specific stromal cells or by supporting the body's own immune system in their fight against the malignant cells. However, even such approaches often displayed limited responses in PDAC patients [8,9].

In particular the growing understanding of the TME points out a different challenge in the development of efficient therapeutics – a lack of biologically relevant *in vitro* and *in vivo* models that resemble the complexity of PDAC observed in patients. Although recent advances in drug development are the use of three dimensional (3D) *in vitro* tissue models to better replicate the complex architecture and cell–cell interaction found *in vivo*, such models mostly lack the inclusion of the TME in a relevant fashion. *In vivo* models on the other hand continuously face the challenge to recapitulate a human-like tumor development and progression. This includes genetic mutations found in PDAC, immune escape, invasion and metastasis, while being a reproducible and feasible experimental method to allow testing therapies [10]. However, animal models have been continuously improved to mimic the development of PDAC originating from genetic mutations representing a realistic progression and phenotype of PDAC found in humans [10]. In addition several different strategies have emerged in the recent decade to design 3D *in vitro* models that allow for a more specific and better evaluation of novel therapeutics before embarking on animal models [11].

In this review, we provide an overview of the current *in vitro* and *in vivo* models replicating the complexity of PDAC in a relevant fashion to assist the development of novel efficient therapeutics against PDAC. Hereby, we will particularly focus on the complexity of current *in vivo* models and the challenges current 3D *in vitro* models face to reach the same level of complexity, while offering high control on the system itself. Furthermore, we will discuss how 3D *in vitro* models can currently aid in and improve current drug development processes that are often based on simple evaluation of novel therapeutics in 2D-based cell assays before directly embarking on sophisticated animal models. In this review, we first will briefly describe the composition and function of the TME in PDAC given its importance in PDAC progression. Next, we will discuss different *in vitro* tumor models

replicating PDAC by using cell-based and engineering-based methodologies and highlight how such models are used in the evaluation of novel drug candidates. We will then focus on novel developments in *in vivo* models that try to mimic the human PDAC phenotype and discuss advantages and disadvantages of such models for drug development. Finally, we will describe alternative models that aid in drug development such as computational models before concluding with a final perspective and future challenges.

2. Key features of PDAC tumors

Commonly, PDAC arises from the head of the pancreas and is often related to genetic mutations leading to the activation of the oncogene KRAS and the inhibition of tumor suppressor genes TP53, p16/CDKNA2A, SMAD4 and BRCA2 [12–14]. Although several subtypes of PDAC exist, it predominantly shows a glandular pattern with the characteristic duct-like structures giving PDAC its name [12,15]. Based on histopathological studies, 3 different precursor lesions of PDAC have been identified: Pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN), where the majority of invasive PDAC develops from PanINs (Fig. 1**A**) [12,16]. Based on their degree of dysplasia, these lesions can be divided into 3 grades, PanIN-1, PanIN-2 and PanIN-3 [17]. As it has been shown that genetic alterations already occur in early stages of PanIN, therapeutic intervention at these stages should provide the highest chance of cure. Unfortunately, PDAC is asymptomatic for a prolonged time, often resulting in late diagnosis and therefore limited treatment options in patients [18,19].

As PDAC develops it also gradually changes the environment surrounding it leading to the formation of the TME. One of the early definitions of tumors made by Harold F. Dvorak in 1986 states that tumors are "wounds that never heal" and further describes the development of the TME as "wound healing gone awry" [20,21]. Despite being made nearly 35 years ago, these definitions still describe the underlying nature of the TME in a fitting way. Originally tumor cells cause an inflammatory response in the surrounding stroma resulting in a wound healing response in the tissue. Next, tumor cells can gradually change the stroma, at least in part, towards a tumor supporting environment that itself is characterized by an anti-inflammatory behavior eventually supporting the tumor in its growth and resistance to therapy and immune clearance.

2.1. Components of the PDAC microenvironment

In PDAC, the TME represents a complex and dense tissue consisting of cancer-associated fibroblasts (CAFs) accounting for around 80% of the tumor stroma, alongside tumor-associated macrophages (TAMs), neutrophils, infiltrating regulatory T cells (Tregs) and natural killer (NK) cells. In addition there is a high abundance of tumor -associated extracellular matrix (ECM) (Fig. 1**B**) [3,22]. The interaction of these stromal cells with tumor cells and autocrine interactions can play a crucial role in the growth and metastasis of PDAC. Next to that it has significant influence on the immune evasion and resistance to chemo- and radiotherapy as extensively discussed elsewhere [3,22–25]. The high resistance of PDAC to current treatment is believed to be based on several factors including the presence of the dense tumor stroma, suppression of the adaptive immune system, the lack of the so-called enhanced permeability and retention effect (EPR) as well as the collapse of vasculature within the TME.

2.2. Dense tumor stroma

In particular the abundance of CAFs, which in PDAC largely originate from pancreatic stellate cells that upon activation by cancer cells attain a myofibroblast-like phenotype, display high similarities to a conventional wound healing response in tissues. CAFs are the key players in the desmoplastic reaction characteristic of PDAC due to their production of high amounts of ECM proteins such as collagen, fibronectin, hyaluronic acid and through their ability to form a direct physical barrier for the penetration of therapeutics. Recently, novel insights into the PDAC TME led to the identification of different types of CAFs within the TME. Besides myofibroblasts-like CAFs (myCAFs), which are usually defined by a high expression of alpha-smooth muscle actin (αSMA), the TME also comprises of CAFs that secrete inflammatory cytokines such as interleukin (IL)-6, so-called inflammatory CAFs (iCAFs). A third subset are CAFs that seem directly involved in antigen presentation and are characterized by a high expression of major histology complex class II (MHCII) (apCAFs) [26,27]. These early findings indicate that "not all CAFs are equal" and we are just beginning to understand the complexity of various (yet to be identified) CAF subsets in tumor progression and metastasis.

2.3. Blood vessel collapse and lack of EPR effect

The desmoplastic reaction, accompanied by excessive ECM deposition, leads to a high intratumoral pressure, preventing therapeutics from entering the tissue. Furthermore, it causes the collapse of intratumoral and surrounding blood vessels, preventing treatments from reaching the tumor site in the first place [28,29]. A crucial concept in the treatment of solid tumors such as PDAC is the presence of the so called EPR effect. The EPR effect describes the accumulation of delivered drugs in tumor tissues in a higher amount compared to healthy tissues based on the presence of leaky and abnormal vasculature in tumors as well as an immature lymphatic system [30]. Hence, a therapeutic that is able circulate for longer periods of time throughout the body by for example encapsulating this therapeutic in a nanocarrier system, has higher chances and time to extravasate and accumulate in tumor tissues. The presence of the high desmoplasia and the resulting collapse of blood vessels in PDAC causes also a lack of sufficient EPR effect. As the EPR effect is considered as one main mechanisms for the extravasation of therapeutics the lack of such an effect can drastically reduce the efficacy of these treatments. Although leaky blood vessels are present in PDAC, it is widely considered that the EPR effect does not play a significant role due to the high stroma and blood vessel collapse, which further prevents therapeutics from reaching the tumor area [31,32]. Recent studies have shown that the re-opening of blood vessels by inhibiting the activation of pancreatic stellate cells (PSCs) towards CAFs within the tumor stroma can increase treatment efficacy, which can be mainly attributed to a higher drug perfusion but potentially also to an increase in EPR effect rendering it a promising treatment approach in PDAC [33].

2.4. Immunosuppression

Besides the several physical barriers for therapeutics to overcome, the TME in PDAC is also highly immunosuppressive rendering host anti-tumor responses ineffective [7,23]. Especially the presence of TAMs, which promote tumor progression and invasion, and the lack of functional $CD4^+$ and $CD8^+$ T cells have been shown to be the characteristic of the PDAC TME. As a result, different immunotherapies have been applied in recent years to try to re-modulate the immunosuppressive environment and reactivate the tumor killing potential of the immune system [34].

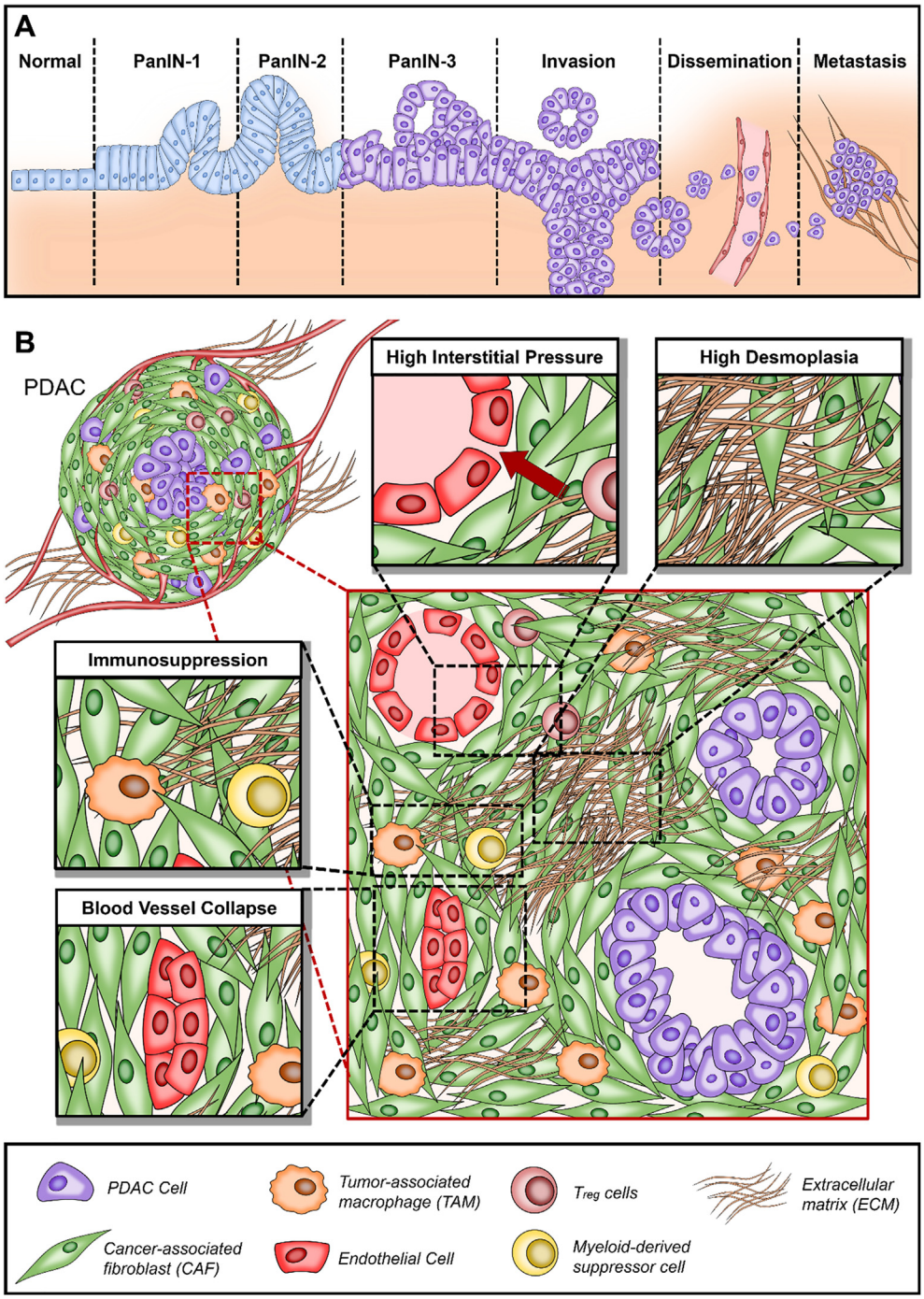

Fig. 1. Development and characteristics of PDAC. A) Schematic representation of the development of PDAC describing different PanIN lesions towards invasive PDAC and metastasis. B) Schematic representation of the PDAC microenvironment including different cell types and highlighting PDAC characteristics that play a significant role in drug efficacy.

To create tumor models that closely mimic the biological characteristics of PDAC in patients, it is crucial to implement all components of the TME. In such a way PDAC models should not only allow for the evaluation of novel drug candidates in a realistic environment but also facilitate the further understanding of cell–cell and cell-ECM interactions within the TME that are not yet fully understood. Such biologically realistic models can also be used to identify novel markers or targets for therapeutic intervention in the future.

3. *In vitro* culture models

The use of *in vitro* models forms the first line of evaluation to investigate if a novel drug candidate displays the hypothesized efficacy against its specific target. In the last decades, *in vitro* models were often based on the culture of a 2D monolayer of the targeted cell type, mostly epithelial tumor cells [35–37]. To date, this method still remains the most common way of evaluating the efficiency of drug candidates. However, with the increasing

understanding of the complexity of tumors, *in vitro* models have experienced a rapid change towards more biologically relevant models, sometimes including the specific TME. In this section the evolution of *in vitro* models from simple 2D monocultures towards multi-cellular 3D models will be discussed and how these models can be applied for the development and evaluation of novel drug candidates.

3.1. 2D in vitro models of PDAC

As aforementioned, the 2D monolayer culture of cells still remains one of the most commonly used techniques to investigate the efficacy of novel drug candidates *in vitro*. As a result, multiple PDAC derived cell lines have been established in recent years, which are currently commercially available. For example, around 15 different immortalized pancreatic cancer cell lines are currently listed in the American Type Culture Collection, each with unique characteristics as well as advantages and disadvantages [38]. Although these cell lines are commonly considered stable based on their immortalization, and are can therefore be propagated without limit in theory, it should be taken into account that even immortalized cell lines display changed characteristics after several number of passages. In particular, potential cross-contamination, mycoplasma infection, miss-identification and genetic mutation might alter the behavior of these cells [39–41]. It has been shown that different passages of the same cell line responded differently towards chemotherapy depending on their passage number [42]. As a result, such cell lines, despite their frequent use, should be handled with care, especially in drug screening and development applications. Besides commercially available cell lines, primary patient-derived cells and the use of induced pluripotent stem cells has been an attractive strategy to extent the available cell culture models with more patient-specific cell types. For instance, Kim *et al.* reprogrammed PDAC cells from patients towards pluripotent stem cells that can be used to study early PanIN stages [43,44]. Despite the high-throughput and cost-efficacy of cell lines, the limitation to culturing a single cell type and the resulting lack of stromal interactions poorly reflects the actual *in vivo* situation. Furthermore, the absence of ECM, oxygen and nutrient gradients or relevant cell–cell and cell-ECM interaction, as well as potential changes in the behavior of cell lines based on *in vitro* passage cycles, renders these models insufficient to display the complex biological features observed *in vivo*. Furthermore, it should be taken into account that a validation of a novel drug compounds on cancer cells only, is not sufficient to fully assess the specificity of these compounds to cancer cells. In addition to cancer cells, novel therapeutics also need to be evaluated on healthy cells to demonstrate the specificity of the therapeutics towards cancer cells as well as low cytotoxicity towards other cells. To mimic the realistic situation a co-culture of cancer and non-cancer components is crucial to assess the efficacy and specificity of novel therapeutics.

3.1.1. Transwell assays

One strategy to mimic the interaction between cells in tumors is the use of Transwell inserts. First introduced in 1986, Transwell inserts are composed of a porous polymeric membrane that is placed into conventional well plates, and allow the culture of two different cell types to study their paracrine crosstalk and investigate (induction of) cell migration [45,46]. Introducing a thin layer of hydrogel, e.g. Matrigel, on top of the membrane, allows for investigating the invasive behavior of cells towards paracrine stimuli [47]. Chen *et al.* studied the effects of Mucin-20 (MUC20) in cancer cell invasion using Transwell systems. The human PDAC cell lines HPAC and HPAF-II were cultured together with PSCs or PSC conditioned medium and the migration and invasion of the PDAC cells was studied [48]. A clear inhibition in the migratory and invasive behavior of MUC20-deficient HPAC and HPAF-II was observed compared to the wild-type cells. Similarly Yan *et al.* recently demonstrated by co-culturing cancer cells and PSCs in a Transwell setup, that the inhibition of ERK1/2 by SCH772894, a novel ERK1/2 inhibitor, in PSCs can significantly inhibit the PSC induced migration of metastatic PDAC cells, while treatment of cancer cells alone did not demonstrate significant inhibition after treatment [49].

Transwell models represent arguably one of the simplest models to study interactions between cancer cells and TME components and show potential to investigate the effects of inhibitory therapeutics. Although recent developments in material science allows for the fabrication of porous membranes that display semi-3D structures, more closely mimicking the microvilli found in the gut, they still rely on 2D culture of cells [50]. Despite these limitations, Transwell models are still widely used due to their simplicity and reproducibility, allowing for rapid screening of novel compounds. The future implementation of 3D architecture might lead to more biologically relevant models, while leaving the simplicity and robustness intact.

3.2. Ex vivo models of PDAC

A different approach to use biologically relevant models for evaluating novel drug candidates for PDAC is the direct use of biological tissues obtained from PDAC patients. In 1985, Smith *et al.* introduced the use of "precision-cut" tissue slices obtained from PDAC patients, which, as the name implies, are cut in with well-defined parameters to be used for drug screening *in vitro* [51]. Precision-cut slices allow for the evaluation of therapeutics in a biological-relevant context, which consists of the tumor cells, the respective TME components, vasculature and the ECM [52–55]. Recently, Misra *et al.* demonstrated the use of precision-cut PDAC tissue slices in a laboratory setting [56]. They obtained PDAC tissues from patients that underwent surgical resection and cultured 350 μm slices for up to 4 days. These slices displayed the structural integrity, phenotypic characteristics and functional activity found in PDAC. They further demonstrated the application for drug screening by showing the responsiveness of these slices towards rapamycin, a mTOR inhibitor, which caused a substantial reduction in pS6 levels compared to untreated control tissues. Very recently, the same group evaluated their culture methods by investigating changes in the transcription patterns using genome-wide transcriptome profiling, comparing freshly isolated cuts with cuts that have been cultured for several days. They found that genes related to hypoxia and angiogenesis (HIF1α and VEGFA, respectively) were significantly increasing with longer culture times, demonstrating how the culture of precision-cut slices can influence potential therapeutic outcomes [57]. Such precision-cut slices are especially interesting for drug development as they are capable to preserve the tissue architecture found in PDAC. Nonetheless, precision-cut slices also display several disadvantages. The limited time these slices remain viable *in vitro* hampers the screening of therapeutics that require longer incubation times to show anti-tumor effects. Furthermore, only 10–20% of PDAC patients are suitable for surgical resection of PDAC, limiting the overall availability of tissues and creating a selection bias [58]. In addition, the tumor tissue will be highly heterogenous in nature, even when obtained from a single patient, which can limit the reproducibility and general applicability. This heterogeneity in precision-cut slices can be beneficial when predicting the efficacy of therapeutics in different patients but largely to examine the efficacy at the late stage. However, in the early drug development usually more reproducible and standardized models are required to fully understand the mechanism of action of a novel drug.

3.3. 3D in vitro models of PDAC

The advancements in biomaterial science, biofabrication and microfluidics over the recent decades, facilitated the generation of complex cell culture models that are capable of mimicking the tissue architecture found *in vivo* (Fig. 2). One of the major advantages compared to conventional 2D culture models is the focus on implementing a controlled 3D aspect that mimics the tissue architecture. In general, two different approaches to create 3D *in vitro* models have emerged more or less in parallel in the recent years: (i) cell-based approaches, including the use of 3D spheroids and organoids [36,59–63], and (ii) engineering-based approaches, mainly involving scaffold-based and 3D bioprinted models as well as tumor-on-chip platforms [45,64–66]. Whereas cell-based approaches mainly rely on the natural capability of cells to develop and organize themselves into 3D structures, engineering-based approaches are mainly driven by a specific goal, which defines the overall structure and composition of the model. Independent of the approach, a 3D *in vitro* models for PDAC have to fulfil several criteria to fully mimic PDAC in a biologically relevant fashion as well as to present a platform that is highly suitable for drug screening and development as well as allows to investigate and understand cellular and acellular processes in PDAC. First, 3D *in vitro* models for PDAC have to replicate the characteristics found in PDAC patients as previously described. In particular, such models have to implement the characteristically high stroma of PDAC, which is mainly produced by CAFs, the immunosuppressive environment and the mechanical properties to fully mimic the situation of PDAC *in vivo*. Second, while replicating the PDAC in a biologically fashion, 3D *in vitro* models in general have to offer a high reproducibility to function as suitable platform for drug screening and development. Furthermore, while trying to mimic the complexity of PDAC, such models have to allow for a comparably simple and cost-effective read-out to display a clear advantage over animal models. Additionally, novel 3D *in vitro* models should offer a high-throughput similar to conventional 2D platforms, to allow testing and validation of novel drug compounds in a fast and effective manner. Several different 3D *in vitro* models for PDAC have been development in the recent years, each with its unique characteristics as well as advantages and disadvantages (Table 1). These models will be discussed in more detail in the following sections.

3.3.1. Spheroid-based culture models

3D spheroids are one of the main representatives of cell-based *in vitro* models that do not rely on excessive external cues such as the addition of specific growth factors or a scaffold material. Over the years several different spheroid culture techniques have been developed including suspension-, liquid overlay- or low-adherent surface or hanging-drop cultures, magnetic levitation and microfluidic culture approaches as extensively discussed elsewhere [67–71]. Despite the wide variety of techniques, the general principle to generate spheroids is common for all approaches. This is mainly based on the cell's own capability to form 3D cell aggregations that are held together by cell–cell contacts and the presence of ECM that is produced by the cells themselves. Based on the dense environment that is generated, spheroids often show nutrient- and oxygen gradients similar as observed in the *in vivo* situation. The capability to create these dense structure makes 3D spheroids highly interesting for modelling PDAC, which is characterized by the presence of a dense stroma. Furthermore, PDAC spheroids can contain different sub-populations of cells, which is not feasible when cells are cultured in 2D. For instance, it has been recently shown that spheroids based on different pancreatic cell lines or primary cells isolated from, for example, KPC mouse tumors include subpopulations of cells with cancer stem cell (CSC)-like properties, such as a high expression of CD44 [72,73]. The presence of these CSCs in the spheroids was related to a higher tumorigenic potential of these spheroids as well as significantly increased resistance to different chemotherapies, including gemcitabine, carboplatin and paclitaxel. The formation of a dense stroma, different subpopulations of cells as well as overall higher

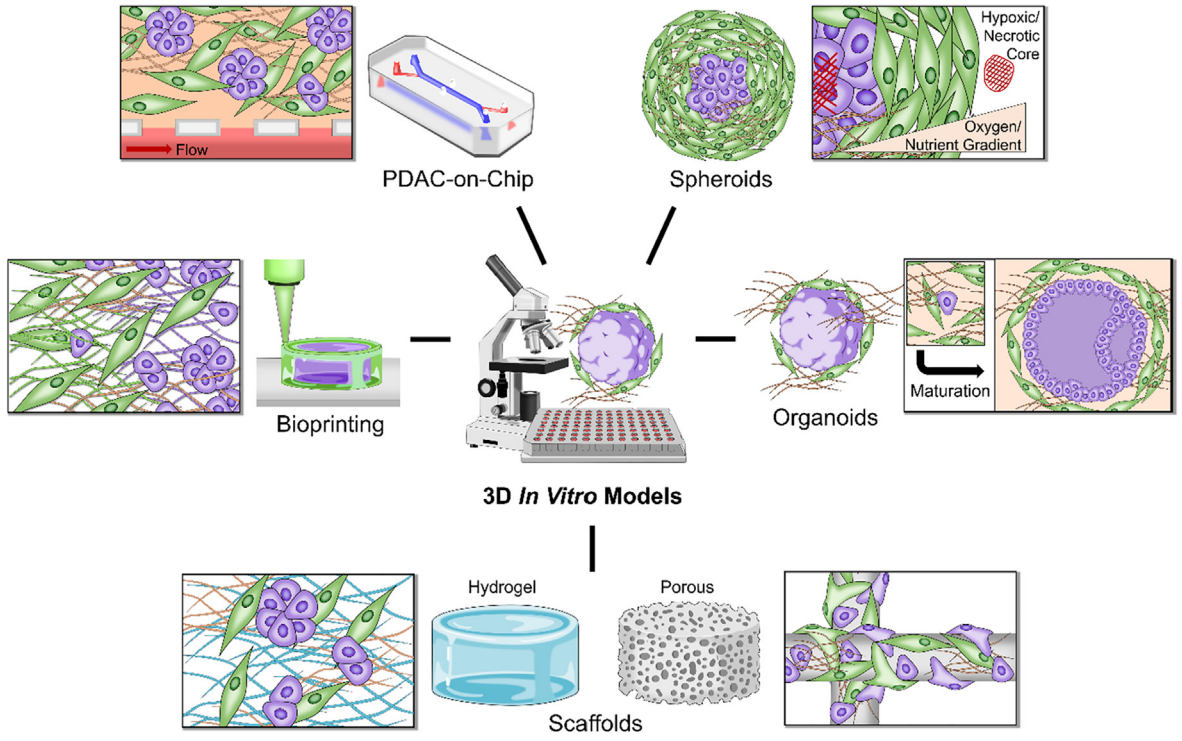

Fig. 2. Overview of different 3D *in vitro* models highlighting design characteristics for each model.

Table 1
Overview of different *in vitro* models used to study and mimic PDAC describing their characteristics, advantages and limitations.

In vitro Models	Characteristics	Advantages	Limitations
2D cultures	Culture of cells in a well plate/petri dish, often tissue-culture treated polystyrene plates	• Simple • Low cost • High-throughput • Standardized • Commercially available	• 2D flat culture • Limited co-culture possibilities • Limited ECM production • High stiffness of surface • Enhanced drug/nanomedicine exposure, no drug penetration
Transwell cultures	Cells seeded onto a membrane placed into a well, allowing to non-contact co-cultures	• Simple seeding & analysis • Commercially available platform • Standardized • Allows study of paracrine signaling	• 2D flat culture • No direct co-culture of cells (juxtacrine signaling) • Limited ECM production • Different surfaces, membrane vs. plastic • No possibility to assess drug penetration
Ex vivo tissue slices	Tissue slices obtained from animals or patients, often in form of precision cut slices to be cultured in a lab environment	• Biologically highly relevant architecture and composition	• Highly heterogeneous, limited reproducibility • Usually short culture times • Fast loss of cellular phenotypes due to culture conditions • Limited availability
Spheroids	(Forced) aggregate of multiple cells, driven by cell–cell attachment and ECM production	• Simple & high-throughput • Allows for co-culture of multiple cell types (e.g. tumor cells & TME) • Production of relevant ECM by cells • Presence of oxygen & nutrient gradients • Suitable to study drug/nanomedicine penetration • Scalable for commercial purposes	• Limited number of cells (usually maximum of 2–3 cell types) • Limited amount of cell types suitable for spheroid culture • Some cultures require additional aggregation enhancers (e.g. Matrigel) • No control on cellular arrangement
Organoids	Originates from single cells following natural developmental stages, usually cultured in a hydrogel environment (e.g. Matrigel)	• Follows developmental stages similar to actual organs • Allows for co-culture of multiple cell types (e.g. tumor & TME (though limited at the current stages)) • Highly relevant morphology & phenotype • Suitable to study drug/nanomedicine penetration	• Establishment can take long (up to several months) • No standardized protocols yet • Often based on a given hydrogel environment (e.g. Matrigel) • No control on cellular arrangement and growth • Limited in size
Scaffolds/ Hydrogels	Crosslinked network of natural or synthetic polymers, usually shaped by the use of a mold/template	• Simple & based on standardized protocols • Gel/scaffold material often commercially available • Cultures can be large in size (mm/cm range) • Possibility for oxygen & nutrient gradients	• Often limited to the culture of one cell type • 3D conformation requires a mold to form • No control on cell arrangement • Materials used often do not fully replicate the *in vivo* ECM • Large models need extra perfusion
3D Bioprinting	Controlled layer-by-layer deposition of cells (often embedded in a hydrogel) following a computer programed design	• Cells can be positioned in a precise and reproducible manner • Allows for the printing of gradients/complex shapes within the same models (e.g. desmoplastic regions) • Different printing strategies available • "Plug & play" platforms and materials increasingly commercially available	• Time-consuming, limited throughput • Printing process can decrease cell viability/performance • Initially cost intensive (3D bioprinter, trainings etc.) • Large models need extra perfusion • Often rely on hydrogel carrier with limited biological relevance to *in vivo* ECM
PDAC-on-chip	Cells cultured with a designed chip (based on e.g. PDMS), often including multiple channels and chambers. Usually combined with a fluid flow.	• Very controlled environment • Small volumes required • Simple readout due to optical transparency and small size allows to apply flow in a controlled manner • High reproducibility • Commercial platforms available	• Limited throughput, especially when combined with flow • Requires special equipment (e.g. pumps) and, depending on design, access to special facilities (e.g. clean room) • Commonly used material for chips (PDMS) is hydrophobic and can absorb drugs

chemoresistance facilitates the use of spheroids for drug screening applications [33,74,75]. The cell-driven formation without the need for external cues allows for the generation of a high number of spheroids, which facilitates high-throughput evaluation of novel drug candidates. In the last years, several different 3D (multicellular) spheroid culture systems have been developed that aim to mimic the dense stroma-rich structure found *in vivo*.

3.3.1.1. Heterospheroids including two cell types. Recent 3D PDAC spheroids often include CAFs to display a more *in vivo* representing multicellular culture [75,76]. So-called PDAC heterospheroids are often generated by combining pancreatic cancer cells with pancreatic stellate cells, which transform into CAFs after close contact with the tumor cells. For instance, Priwitaningrum *et al.* developed Panc-1/PSC heterospheroids based on the forced aggregation of

cells into microwells using centrifugation [75]. After 48 h they observed the formation of heterospheroids which were further evaluated for their application in drug screening by incubation with silica nanoparticles. A significantly reduced penetration of nanoparticles in the presence of CAFs was observed demonstrating a similar barrier function as found *in vivo*. Later these Panc-1/PSC heterospheroids were used to evaluate the efficacy of novel therapeutics before embarking on animal studies. They found a similar reduction in heterospheroid growth *in vitro* and tumor growth *in vivo*, where tumors were based on the subcutaneous injection of the same cells, demonstrating the potential of such heterospheroids to predict the efficacy of novel drug candidates [33,77]. Recently, Panc-1/PSC spheroids have also been used in the evaluation of novel therapeutics such as microbubbles and super paramagnetic iron-oxide nanoparticles (SPIONS) [78,79].

3.3.1.2. Multicellular spheroids. The comparably simple construction of heterospheroids also facilitates the inclusion of additional cell types. For example, Lazzari *et al.* introduced human umbilical vascular endothelial cells (HUVECs) into their PDAC spheroids composed of Panc-1 cancer cells and MRC-5 fibroblasts to mimic vascular components found *in vivo* [80]. They were able to show an increasing chemoresistance when CAFs and HUVECs were included into the heterospheroids. Although the implementation of HUVECs did not result in the formation of blood vessel-like structures, their study is one of the first to implement multiple stromal cells within a spheroid model.

3.3.1.3. Advantages & current limitations. Despite the simplicity of generating them and the potential to include stromal components, spheroids face several hurdles when reproducing the complexity of human PDAC. A major challenge is the lack of an overall control on the spheroid architecture. In particular, heterospheroids of cancer cells and fibroblasts can face the problem that fibroblasts tend to aggregate faster and more tightly compared to cancer cells. This can eventually results in a hetero-spheroid composed of a fibroblast core surrounded by cancer cells [75]. However, the opposite pattern is present *in vivo*, where epithelial cancer cells are surrounded by a dense fibroblasts-rich stroma. Nevertheless, the direct contact and crosstalk between the cells still presents a more relevant *in vitro* model compared to a 2D cell culture experiments or single cell spheroids. Another disadvantage is the limitation to cell types that display high cell–cell attachment and/or produce sufficient ECM to aid in cell aggregation. In such way the inclusion of macrophages, for instance, into these cultures is challenging as these cells are more prone to degrading ECM rather than producing it, limiting their direct introduction into heterospheroids [81]. Kuen *et al.* recently presented a strategy that allows the inclusion of macrophages into PDAC spheroids by allowing monocyte-derived macrophages to infiltrate pancreatic cancer cell/fibroblast heterospheroids post-formation [82]. They found that macrophages were able to infiltrate the tumor heterospheroids and adopted a tumor-associated phenotype based on the upregulation of M2 macrophage markers, similar to TAMs *in vivo*. In this way they generated multicellular spheroids consisting of tumor cells, CAFs and TAMs. However a major disadvantage of this approach, is the lack of control on the number of macrophages that infiltrate the spheroids, potentially hampering the reproducibility of these models, which should be solved in the future. Despite these limitations, (hetero)spheroids are one of the simplest 3D *in vitro* models to evaluate novel PDAC therapeutics in a high-throughput manner that is available for a broad research community without the need for excessive experience of 3D *in vitro* culture.

3.3.2. Organoid-based culture models

In recent years organoids have received a lot of attention due to their potential to develop 3D organ-like structures that resemble key biological features and relevant tissue development [60,61]. The term organoid describes a 3D tissue that aims to mimic organ-like structures and functions and is not entirely new, since it was first reported by Smith and Cochrane in 1946. They reported the culture of self-arranged 3D cell clusters such as spheroids already. However, the term organoid was re-defined in the last ten years [60,61,83,84]. Although still spheroidal in shape, organoids significantly differ from spheroids as they develop from single cells. These cells are mainly induced pluripotent stem cells (iPSC) or adult stem- or tumor cells, that are encapsulated into a hydrogel environment (e.g. Matrigel). In this way 3D organ-like structures can be generated that follow similar developmental steps as natural tissues, representing tissue-specific morphology and architecture.

3.3.2.1. Engineered and patient-derived cancer organoids. In general, cancer organoids can be classified into two subclasses [85]: (i) Engineered cancer organoids (ECOs), which are based on iPSC or adult stem cells to form an healthy tissue organoid before being genetically edited towards a tumor organoid, and (ii) patient-derived cancer organoid (PDOs), which are based on tumor cells obtained from patients either by surgery/biopsy or from circulating tumor cells. As ECOs originate from healthy tissues, these organoids are often applied in carcinogenesis research but due to the complexity of the genetic editing and culture strategy are not widely used for drug screening purposes. PDOs, on the other hand, form a suitable platform for rapid screening of (personalized) medicine due to their tumor origin and relatively low culture duration/efforts [60,61,85]. As a matter of fact, these characteristics make PDOs also highly interesting as an alternative to patient-derived xenografts, which are usually more expensive and labor-intense and require the use of lab animals [86]. Due to their potential for drug development applications, this review will primarily focus on PDO models to mimic the PDAC environment.

Similar to spheroids, pancreatic PDOs initially focused on the monoculture of pancreatic cancer cells as novel organoid-based 3D models. As one of the first, Tuveson, Clevers and co-workers generated pancreatic PDOs from low-grade PanIN tissues from Pdx1-Cre; LSL-$Kras^{G12D/+}$ (KC) mice. These organoids were cultured in a Matrigel environment and medium containing different growth factors such as transforming growth factor β (TGFβ) pathway inhibitors A83-01 and Noggin or Wnt family member 3a (Wnt3a) [87]. The organoids displayed *in vivo*-like architecture, including the characteristic ducts of PDAC as well as continuous growth of the cultured organoids. Due to the increased understanding of the importance of the TME in PDAC at the time of these studies, increased efforts were made to investigate the potential of the co-culture of pancreatic PDOs with TME components to achieve a more biologically relevant close-to-patient model.

3.3.2.2. Co-cultured organoids. The same group around Tuveson, Clevers and co-workers explored the culture of the pancreatic PDOs with PSCs that were embedded in Matrigel [26]. They found that PSCs in the model were activated and differentiated towards different types of CAFs, depending on their contact with tumor cells. In direct contact PSCs displayed the conventional myCAF phenotype, while distant PSCs, which mainly rely on paracrine crosstalk with the pancreatic PDOs, displayed an inflammatory phenotype, later classified as iCAF. Both CAF types significantly affected tumor growth and invasion in the organoid model. The identification of these novel CAF subsets using this organoid model demonstrates how they can be used to investigate biologically relevant processes and interactions in greater detail. In particular, the

co-culture of PDOs with TME components is not limited to the tumor-CAF co-cultures but can be extended to most cell types within the TME. For instance, Choi *et al*. recently co-cultured PDOs from PDAC patients with human umbilical vein endothelial cells (HUVECs) to study their crosstalk [88]. They reported that endothelial cells play a crucial role in the maintenance of $CD44^+$ cells, particularly orchestrated by the Wnt and Notch pathways, which further demonstrates the potential of PDOs to study underlying biological mechanisms within the PDAC TME.

3.3.2.3. Organoids for personalized medicine & drug screening. The ability to grow PDOs from patient tissues makes them highly interesting to explore personalized medicine. Personalized PDOs can aid in the prediction or how patients react to certain treatment and might help to identify the most promising treatment strategy. Tiriac *et al*. demonstrated the use of PDOs for such drug screening applications by the generation of a pancreatic PDO library consisting of organoids derived from 66 PDAC patients to serve as platform for genetic profiling as well as drug screening [89]. They identified novel common genetic alterations in these organoids that serve as driver oncogenes in PDAC and demonstrated "patient-like" responses towards standard of care PDAC therapeutics such as gemcitabine, nab-paclitaxel, irinotecan, 5-fluorouracil and oxaliplatin. In such way, PDOs could be used to predict the efficacy and sensitivity of treatments as well as aid in the nomination of alternative treatments for patients displaying a similar genetic make-up of mutations as found in the representative organoids. These findings represent one of the first approaches to use PDOs to assess drug sensitivity and efficacy in patients in a fast and efficient manner, in particular compared to, for instance patient-derived xenograft *in vivo* models, which require longer and more complex generation.

One of the major drawbacks in this approach, is the lack of TME components such as CAFs that can strongly influence the efficacy of PDAC-targeting drugs. Furthermore, similar to the aforementioned spheroid cultures, most of the current PDO-based culture approaches lack the presence of immune components. Recently, Tsai *et al*. presented an approach to combine PDOs with fibroblasts and incorporated lymphocytes to study the penetration of T cells into the TME [90]. They co-cultured human PDOs with patient-derived fibroblasts in a hydrogel environment while letting lymphocytes, which are suspended in the culture, penetrate into the organoid. The group demonstrated that the co-culture of PDOs with fibroblasts lead to the activation of these cells towards myCAF, which eventually had significant influence on the efficacy of gemcitabine on tumor cells. Furthermore, they observed the penetration of pre-polarised $CD4^+/CD8^+$ T cells, mimicking the tumor-targeting infiltration of these immune components into the TME, which demonstrated the use of PDO models to investigate the efficacy of immunotherapies such as checkpoint inhibitors.

3.3.2.4. Advantages & current limitations. Although, an increasing number of studies show the incorporation of crucial TME components into PDO models, more sophisticated models are required to mimic the patient-specific environment in PDAC patients in a biologically- and therapeutically relevant fashion. For example, the incorporation of other immune cells, such as macrophages and neutrophils, forms a crucial aspect for the generation of a PDAC relevant immunosuppressive environment. The inclusion of these components facilitates the investigation of the efficacy of novel immunotherapies in such models. A different challenge in the use of PDO models is the lack of standard protocols for the culture of organoids as well as a reported high heterogeneity within these models, which can limit the suitability for drug development applications, as mentioned earlier [60,61,87]. Furthermore, depending on the culture conditions and the need for different growth factors, organoid cultures can be expensive compared to for instance spheroid cultures. Despite these disadvantages PDO-based culture systems have demonstrated their potential to investigate biological mechanisms with high relevance to the *in vivo* situation as well as being suitable for (personalized) drug screening rendering them a highly valuable tool.

3.3.3. Scaffold-based culture models

As previously mentioned, scaffold-based culture models are one of the fundamental engineering-based 3D culture approaches. In general, a scaffold can be defined as a man-made biomimetic environment that provides cells with the natural architecture and stimuli they would experience *in vivo* [36,91,92]. Despite being extensively described and discussed in several scientific publications [11,66,74,91], the term scaffold does not have a clear definition. It can describe the use of porous ceramic or polymeric scaffolds, which are often based on poly-lactic acid (PLA) [93], poly(lactic-co-glycolic acid) (PLGA) [94], poly(L-lactic acid) (PLLA) [95] or poly(caprolactone) (PCL) [96]. In addition hydrogels can be used, which are usually crosslinked networks of synthetic or naturally-derived polymers, such as collagen [97,98], fibrin [99,100], alginate [101,102], chitosan [103,104], gelatin [105,106], hyaluronic acid [107,108] or silk fibroin protein [109]. In recent years, the use of tissue-derived biomaterials, such as Matrigel or decellularized ECM (dECM), have also found broad application in the field of tissue- and disease modelling due to the presence of several natural tissue-specific proteins and matrix bound growth factors [92,110–113]. However, based on the natural origin of these biomaterials, Matrigel and dECM usually display a high batch-to-batch variation, high costs or difficult purification procedures, limiting their use in standardized drug screening and development applications. In general, scaffold-based cultures can include any culture approach that uses the aforementioned or similar biomaterials to achieve a 3D cell-laden construct. The following section will only focus on scaffold-based cultures that do not use scaffolds as a growing environment for spheroids or organoids and do not use bioprinting/organ-on-chip techniques as these will be discussed later.

3.3.3.1. Scaffold-based models for PDAC. All scaffold strategies aim to provide cells with the necessary 3D environment where they can attach, migrate and proliferate similar to the *in vivo* conditions. As a result of these characteristics, cancer cells and TME components often display a more biologically relevant phenotype compared to the conventional 2D culture. For instance, PDAC patient-derived cancer cells seeded onto biomimetic porous scaffolds of poly(vinyl alcohol)/gelatin (PVA/G) and poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) [114]. displayed *in vivo*-like morphology as well as a high expression of PDAC-relevant metalloproteinases (MMPs) 2 and 9. This demonstrates the advantage of scaffold cultures over conventional 2D models. More recently this approach was extended by combining PDAC cancer cells, with microvascular endothelial cells and pancreatic stellate cells in a single polyurethane-based scaffold [115]. High cell proliferation over a duration of 5 weeks as well as *in vivo*-like cell rearrangement, mimicking the natural tissue architecture, was observed. Furthermore, these cells produced high amounts of ECM proteins such as collagen and fibronectin similar to the desmoplasia observed *in vivo*. Although these studies did not yet involve drug screening, the use of scaffold-based models for drug screening applications has been reported, demonstrating the suitability and advantage of such models for drug screening applications [66,116].

3.3.3.2. Advantages & current limitations. Scaffold-based culture approaches can be an attractive tool to model PDAC *in vitro* as well as to test and develop novel therapeutics, as a result of their rela-

tively simple construction method, availability of the often commercially used biomaterials, and general biocompatibility of the different materials for long time cultures. However, similar to spheroid models, simple scaffold-based cultures do not allow control on the (tissue/cell aggregate) architecture and therefore heavily rely on the cell self-rearrangement. Furthermore, despite the tissue-like nature of the biomaterials used, a scaffold still remains a man-made environment. This allows for the exact control of, for example, porosity, composition and stiffness, but can also directly alter the cell behavior and phenotype. A high degree of control on these parameters is crucial to mimic the cellular behavior found *in vivo*, since otherwise cells might depict a different behavior, which can directly influence the efficacy of tested drugs. Furthermore, natural tissues are often heterogenic in structure/composition. To mimic such heterogeneity a different engineering technique is required, that will be discussed in the next section.

3.3.4. 3D bioprinted culture models

3D bioprinting is an additive manufacturing method, which has been developed in the last decade and rapidly found broad application in the field of tissue engineering and, more recently, in the field of *in vitro* disease modeling. 3D bioprinting was designed to overcome the lack of architectural control of scaffold-based approaches by offering unprecedented accuracy and precision in arranging cells in a 3D volumetric fashion [11,117]. In recent years, several different bioprinting strategies have emerged, each with their own advantages and disadvantages [11,118–122]. Bioprinting strategies include stereolithography-based bioprinting [123–125], extrusion-based bioprinting [11,116], inkjet-based bioprinting [126,127], laser-assisted bioprinting [119,128] and electrospinning-based bioprinting [129–131]. Regardless of the technique used, 3D bioprinting is the patterning of (cell-laden) biomaterials or cells following a computer-aided design. As such, 3D structures can be achieved that display a controlled heterogeneity that is found in natural tissues.

3.3.4.1. 3D bioprinted co-culture models. 3D bioprinting can aid in generating PDAC cancer models that display PDAC-specific areas of high and low desmoplasia and predefined matrix stiffness closely mimicking the natural architecture of PDAC tissues by bioprinting different pre-defined cell-laden biomaterials (e.g. materials with different stiffness or a different amount of cells) within the same 3D structure. For example, 3D bioprinted breast cancer and PDAC models, where the tumor core is surrounded by endothelial cells and fibroblasts/stellate cells, were reported [132]. The exact localization of these cells around the tumor core was achieved by mixing the respective cells types with an alginate hydrogel carrier, followed by 3D printing in a predefined pattern. By initially crosslinking the alginate using calcium-chloride a stable construct could be generated. Following sufficient secretion of sufficient ECM components to support the 3D structure by the fibroblasts/stellate cells, the alginate was removed to achieve a full scaffold-free culture. The suitability of the 3D bioprinted PDAC tissue for drug screening applications was demonstrated by incubation with gemcitabine showing dose-dependent anti-tumoral activity. Furthermore, the cellular responses towards external cues such as TGFβ, a crucial cytokine in natural PDAC tissue was shown. More recently, Hakobyan *et al.* demonstrated the use of laser-assisted 3D bioprinting for generating PDAC spheroid models [133]. They bioprinted PDAC cells onto a layer of gelatin methacryloyl (GelMA), a gelatin derivative widely used in bioprinting applications, before adding a second layer on top. Lastly, the construct was crosslinked using UV to fully enclose the bioprinted cells into the hydrogel. This model displayed high cell viability as well as the expression of PDAC specific markers. Although TME components or drug screening was not included into this study, their approach demonstrates that 3D bioprinting can also be used to culture spheroids or organoids.

3.3.4.2. Advantages & current limitations. Overall, 3D bioprinting paved the way for novel 3D *in vitro* models that can be more precisely controlled in terms of architecture and composition. This gives 3D bioprinted models a clear advantage compared to spheroids/organoid or simple scaffold-based approaches that mainly rely on cell self-rearrangement. In particular, in PDAC this can facilitate the production of *in vitro* models that specifically mimic the desmoplastic/fibrotic barrier that surrounds the tumor and has significant influence on drug penetration. Furthermore, since 3D bioprinting usually involves the use of cells mixed with a scaffold material, multiple cell types can be easily integrated into bioprinted constructs. This enables the inclusion of macrophages and other immune cells, as has already been demonstrated in 3D bioprinted models of other tumor types [116,117]. Although the technique of producing 3D bioprinted models is still technically challenging using a bioprinter set-up as well as highly time-consuming, novel developments in the field aim to improve the production speeds by for instance using novel stereolithography-based techniques or implementing multiple nozzles to create a higher number of replicates within the same printing time [125,134]. As it is in theory possible to culture any cell type in 3D bioprinted PDAC models, using patient-derived material could form a highly promising tool for personalized medicine as it has been demonstrated in 3D bioprinted cancer models of glioblastoma or liver tissues aimed for regenerative medicine [135–137].

3.3.5. PDAC-on-chip

Another rapidly developing field in recent years are microfluidic-based or so-called organ-on-chip (OOC) platforms. Such OOC platforms aim to mimic fundamental (patho)physiological functions of an organ or tissue in a controlled "chip" environment. This "chip" often has the size of a pen-drive and incorporates engraved structures such as interconnected channels or chambers, which are usually in the range of a few 100 μm to a few millimeter, allowing for cell growth in a very confined and well-defined space. After their initial development in the 1990s and the first publications mentioning "organ-on-chip" in 2007 [138–141], they became rapidly recognized for their potential to understand fundamental pathophysiological processes, as well as therapeutic responses. In particular the high degree of control on the applied mechanical forces, orientation of tissue interfaces, cell types, architecture and gradients makes OOC platforms highly interesting for drug screening. Furthermore, OOC platforms usually use very small amounts of bio- and cellular material as well as liquids, which is especially valuable in the screening of expensive drugs or use of precious patient-derived samples. In general OOC platforms are furthermore combined with bioreactors allowing for a controlled flow of medium within the system, which can mimic the systemic administration of therapeutics in the blood stream as well as the blood flow within organs. Whereas initial OOC platforms were mostly based on 2D culture of different cellular components, recent applications often include a scaffold enabling a 3D architecture of the cultured cells within the platform.

3.3.5.1. PDAC co-culture models on chip. The high level of control and variety of applications of OOC platforms, make them highly interesting for mimicking the natural tissue found in PDAC patients. Drifka *et al.* describe the culture of pancreatic cancer cells surrounded by PSCs in an OOC platform, both encapsulated in a collagen hydrogel to achieve a 3D structure [142]. Channels around the PSCs to mimic the presence of blood vessels in PDAC and to apply therapeutics were also included to the system. Interestingly, they observed *in vivo*-like production of ECM molecules as well as

relevant cell–cell or cell-ECM interactions. As a proof of concept for drug screening applications paclitaxel was tested as a model drug, which demonstrated a dose-dependent reduction of cell viability as well as an overall reduction of the ECM compactness within the model. The use of OOC platforms for this purpose was also evaluated by Beer *et al.* In this study the efficacy of cisplatin on different pancreatic cancer cell lines was evaluated using a microfluidic chip platform [143]. In the future combining such approaches with patient-derived cells can form a fast and high-throughput platform for evaluating the efficacy of potential treatment options while only requiring limited amounts of patient samples (e.g. a biopsy) [137]. A different advantage of OOC platforms is the very confined space of the chip culture, often limited to a few hundred micrometers in height. Compared to, for instance bioprinted tissues, which can reach a few millimeters in height and therefore several layers of cells, the tissues in OOC platforms are often limited to a few cell layers which can be analyzed using microscopy without the need of sectioning the tissues. In addition OOC platforms are often based on polydimethylsiloxane (PDMS) combined with glass, which itself secures a high optical clarity of the chip itself [66]. The limited height and therefore number of cell layers and the optical clarity of the chip enable the imaging of cellular interactions directly within the chip, which facilitates the analysis of PDAC specific processes in greater detail. A recent example of this is a OOC platform consisting of two channels within a collagen hydrogel; One channel representing a cancerous pancreatic duct, being lined with pancreatic cancer cells, and the other representing a blood vessel being lined with HUVECs [144]. This model allowed to observe that intravasation of the pancreatic cancer cells into the blood vessels occurred via ablation of the endothelial cells and invasion into the lumen of the blood vessel, which they also observed *in vivo*. This data further revealed the importance of the activin-ALK7 pathway in this process, demonstrating the potential of OOC platforms to studying and identify novel interactions and provide mechanistic insight into PDAC progression.

3.3.5.2. Advantages & current limitations. Overall, OOC models present a highly attractive platform as results of the high degree of control over the culture conditions within a confined space, the potential to include flow, optical clarity and limited amount of tissue/liquid required. In particular, the use of flow does not only facilitate the application of therapeutics in biologically fashion, but also allows for introducing immune cells into the "blood flow". In such way the infiltration of immune cells from the blood stream into the tumor tissue can be replicated in a highly biologically relevant fashion, outcompeting the regular static conditions of other models. Furthermore, such PDAC on chip platforms when combined with an endothelial layer, allow for studying different processes of intra- and extravasation. In such a way, the extravasation of therapeutics, myeloid cells or lymphocytes can be investigated in greater detail as well as metastatic process can be studied involving initial stages of cancer cell intravasation and movement to distant secondary cancer sites [145,146]. A major disadvantage of current OOC platforms, however, is the use of PDMS to create most platforms. It has been shown that PDMS can absorb small hydrophobic molecules [147]. As most therapeutics in PDAC are hydrophobic, chips that specifically aim for the screening of drug compounds need to be composed of a different material. Furthermore, despite recent developments in the manufacture and commercialization of OOC platforms, it stills requires a lot of expertise and equipment (e.g. pumps, tubing, connectors etc.). Although an increased number of plug & play microfluidic system enter the market, people without a fundamental knowledge of these systems might be discouraged by the initial complexity. Additionally the small volumes and dimensions which are used in the systems require the scientists to be trained to handle these systems and avoid air bubbles or increased shear force, which can directly influence cell viability or behavior given the confined space. Nonetheless, OOC platforms form one of the most promising *in vitro* tools to investigate cellular behavior as well to evaluate drug efficiency in a high-throughput manner based on the low volumes of therapeutics and cellular samples required within the chip, ultimately making these systems cost-effective.

4. Animal models

Despite the numerous possibilities and promising applications of current 3D *in vitro* systems, current *in vitro* systems still lack the complexity to fully replicate PDAC progression *in vivo*. In particular, *in vitro* systems are often limited in the number of cells or cell types that can be cultured and often focus only on the target organ, i.e., the pancreas. Although recent approaches have tried multi-organ-chips, which can mimic the biodistribution and toxicity of a therapeutic *in vivo*, such systems are still juvenile and need further improvements and validation before being able to really recapitulate the *in vivo* situation [148–150]. Furthermore, current 3D *in vitro* systems often focus on controlled lab conditions, e.g., using well-defined cell culture medium for their applications. Despite the tremendous progress in 3D *in vitro* models, they often fail in recapitulating realistic *in vivo* response of living systems. Notably, recent studies demonstrate that different properties and components within the blood can inhibit the efficacy of therapeutics tremendously by for example the formation of a protein corona, describing the adsorption of certain blood proteins onto the surface of an injected nanocarrier or therapeutic, which eventually helps the innate immune system recognize circulating therapeutics [151–154]. Such complex interactions, which are not yet fully understood themselves, are challenging to replicate in an *in vitro* setting, although being crucial for evaluation of the efficacy of a therapeutic agent. Hence, animal models still form a crucial tool in drug development and evaluation.

The first animal model of pancreatic cancer was reported by Wilson *et al.* in 1941, describing that feeding albino rats a diet with 2-acetylaminofluorene induced pancreatic cancer [155]. Later, intensive research in pancreatic animal modeling was initiated because of the increasing incidence of pancreatic cancer [10]. The design of pancreatic cancer tumor models is performed via various techniques, ranging from spontaneous generation of tumors by chemical induction, implantation of tumor cells or tissue orthotopically or heterotopically as well as the manipulation of animal's genetic material by inducing mutagenesis (Fig. 3). In addition to cost, time and skills consideration, the tumor characteristics and research application play a role in the right selection of the most suitable and tailored animal model as each model has its own strengths and weaknesses (Table 2). In order to develop a reliable pancreatic cancer animal models, certain criteria should be met [156]. Ideally, a preclinical model should simulate pancreatic tumor progression process in humans reliably and with high reproducibly with respect to both genetic mutations and the occurrence of progenitor lesions such as intraepithelial neoplasia (PanIN) [10,157,158]. Moreover, the model should be able to recapitulate human tumorigenesis such as target organs of metastasis, chemoresistance, escaping immune surveillance and desmoplasia. Furthermore, the model should enable to measure the therapeutic response in a reliable and reproducible manner. Finally establishing the animal model should be affordable and efficient in respect to labor and time of tumor development [10,156,159].

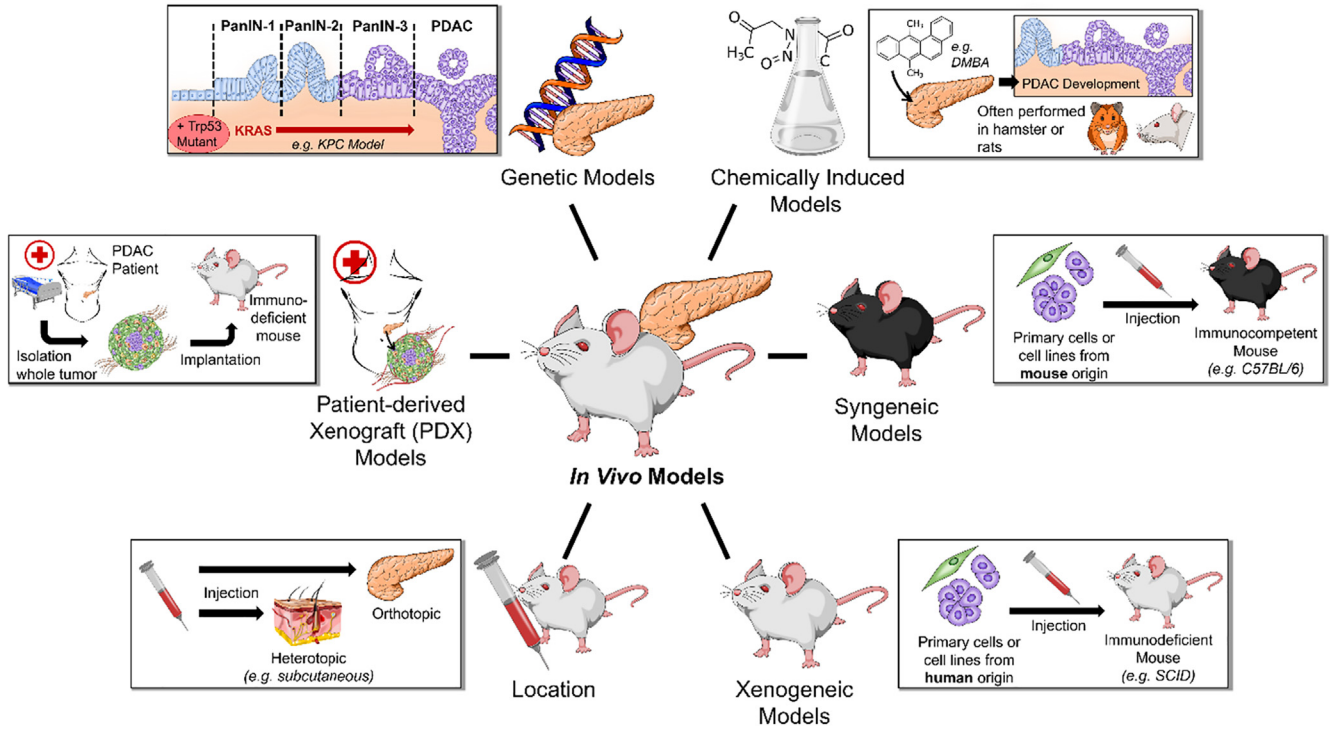

Fig. 3. Overview of different *in vivo* PDAC models highlighting characteristics for each model.

Table 2
The advantages and limitation of *in vivo* models for PDAC.

In vivo model		Advantages	Limitations
Chemically induced		• Immunocompetent • Spontaneous development of tumor	• Genetically irrelevant • Latent tumor development • Inconsistent • Lacks reproducibility • Lacks desmoplasia
Cell line-derived			
A	Syngeneic	• Easy to monitor tumor development • Immunocompetent • Improved mimicking of tumor stroma • Cost-effective • Ability of stromal co-injection	• Cancer cells and hosts are genetically irrelevant to human • Mutation and clonal selection • Low incidence of metastasis (subcutaneous)
B	Xenogeneic	• Tumor cells of human origin • Cost-effective • Ability of stromal co-injection	• Immunocompromised • Costly • Mutation and clonal selection • Lack of spontaneous desmoplasia • Less chemoresistant than PDAC • Low incidence of metastasis (subcutaneous)
PDX		• Tumor vasculature and stroma included • Tumor cells of human origin • Tool for personalized medicine • Genotype is preserved during early passages	• Immunocompromised • Low engraftment rates • Costly • Labor-intensive • Latent tumor development • Gradual replacement of human stroma by murine cells
GEMMS		• Harbours PDAC-oncogenes • Immunocompetent • Early metastasis • Rapid development of tumor • Tumor aggressiveness • Spontaneous desmoplasia • Reproducible	• Latent tumor development • Costly • Difficult tumor monitoring

4.1. Chemical induction of PDAC

Exposure to environmental and chemical carcinogens is known to be a major causative for pancreatic cancer in humans [160,161]. Therefore, it was proposed as one of the ideal methods to generate pancreatic cancer animal models as it has the ability to simulate the spontaneous process of cancer development in humans [162]. However, due to inconsistency, the time-consuming nature of these studies, and lack of reproducibility, other models appear to be more convenient and feasible for research [162,163].

4.1.1. Mice

Although local implantation of 3-methyl-cholanthrene (which resulted in the generation of Panc02 and Panc03 murine pancreatic cancer cell lines) and azaserine was shown to induce pancreatic cancer in C57BL/6 mice and CD-1 albino mice, respectively, murine pancreatic cancer models are not widely developed via chemical exposure due to the diffusive nature of their pancreas as well as their relative resistance to tumorigenesis [163–165].

4.1.2. Rats

As in mice, intraperitoneal injection of azaserine, a DNA-alkylating agent, demonstrated its potential in inducing pancreatic carcinogenesis in rats. Among pancreatic tumor chemical induction methods, azaserine-derived tumorigenesis is considered as the most commonly used model in rats, however many drawbacks are associated with it, as reported earlier [164,165]. In this model, carcinogenesis was reported to emerge from malignant acinar cells lesions rather than ductal transformations, which is a typical feature in > 90% of human pancreatic cancers [159,163,166–169]. Moreover, variable degrees of metastasis in the liver and the lung as well as lymph node infiltration were reported [10,170,171]. Notably, long latency, low incidence of 37.5% and lack of ductal adenocarcinoma are among the significant drawbacks of this model [10,172]. Yıldız *et al.* reported the association of the presence of pancreatic acidophilic atypical acinar cell transformation with azaserine treatment in Wistar rats [173]. Upon aspirin treatment over 5 months, less transformations were reported, suggesting protective roles of Aspirin against acinar cell neoplastic changes [173].

One of the most important carcinogen-induced pancreatic cancer rat models was established by the local injection of crystalline 7,12-dimethylbenzanthracene (DMBA) into the head of the rat pancreas. This model was developed to induce invasive ductal adenocarcinoma, however, it is limited by the lack of *KRAS* oncogenic mutation as well as tumor growth latency, which was reported to range from 5 to 8 months in 80% of treated rats [163,174–178]. Although pancreatic intraepithelial neoplasia (PanIN) was reported to be one of the features in this model [179]. Guo *et al.* identified CXCR7 (C-X-C chemokine receptor type 7) as one of the upregulated markers and therapeutic targets in DMBA-induced pancreatic cancer in rats, and showed that the knockdown CXCR7 *in vitro* suppressed Panc-1 cell invasion and migration [180].

To a lesser extent, other models have been developed using different carcinogens to induce acinar cell pancreatic carcinoma in rats such as nafenopin, N delta-(N-methyl-N-nitrosocarbamoyl)-L -ornithine, 4-hydroxyaminoquinoline-1-oxide or ethyl-alpha-p-ch lorophenoxyisobutyrate [170,181–183]. Overall, rat pancreatic tumor models are rarely reported due to their clinical irrelevance to PDAC with regard to tumor latent development, lack of *KRAS* oncogene, infrequent metastasis, low success rates as well as pancreatic acinar involvement.

4.1.3. Hamsters

In contrast to the highly diffuse pancreas in mice, hamsters possess a compact patterned pancreas that recapitulates the human pancreas to a greater extent compared to mice, recommending hamsters as one of the most suitable animal models for studying chemical-induced pancreatic cancer [184–187]. Furthermore, the hamster pancreas is sensitive to certain carcinogens which are not cancerous in other animals [10]. Due to their selectivity toward pancreas, DNA-alkylating agents N-Nitroso-bis(2-oxopropyl) amine and N -nitroso–bis (2-hydroxipropyl) amine are the most commonly used carcinogens to induce pancreatic cancer in hamsters [188,189]. Notably, these models recapitulate the histomorphological changes that occur in human cancer, including desmoplastic reactions and the characteristic duct-like morphology of PDAC [163,190]. Furthermore, oncogenic *KRAS* mutations are among the most common features in these models, which add to their clinical relevance [191–197]. PanIN lesions were found to be among the earliest neoplastic changes after carcinogen treatment [198]. Additionally, the tumor incidence ranges from 80 to 100% after 2 months of treatment [163]. However, due to their high costs compared to mice, hamster models are not commonly used in preclinical studies. As a result, they are more commonly proposed in preventive, risk-evaluating studies [10,190,199]. For example, as a chemopreventive strategy, a combination of sulforaphane, aspirin and curcumin-loaded nanoparticles was tested in a chemically-induced Syrian hamster pancreatic cancer model and resulted in a significant reduction in tumor incidence compared with the control group [198].

4.2. Xenograft models

Xenograft models involve the implantation of pancreatic cancer cells or tissues into the body of the experimental animal - in most cases mice. They can be classified into orthotopic models, where cancer cells are injected into the pancreatic tissue, or heterotopic models, where cancer cells are injected in a non-pancreatic tissue of the recipient animal, which is subcutaneously in most cases, although intrasplenic and intravenous injection of cancer cells can be used to simulate liver and lung metastasis respectively [200,201]. Orthotopic implantation carries a closer biological relevance than heterotopic models as the tumor develops in its native organ [202]. In contrast to subcutaneous models, and beside the advantage of tumor initiation at the typical site, orthotopic models provide the ability of cancer cell invasion to metastatic sites [200]. To recapitulate the desmoplastic nature of pancreatic cancer in human, fibroblasts or pancreatic stellate cells (PSCs) can be co-injected with cancer cells [203]. However, in other cases, certain types of fibroblast-recruiting cancer cells can be injected alone, with the ability to induce endogenous desmoplasia [203]. Furthermore, cancer cells can be injected in a biocompatible carrier matrix such as Matrigel for more efficient localization of the tumor. Overall, graft models are commonly used due to their short tumor development latency, low cost, reproducibility and easy of response assessment [204].

4.2.1. Syngeneic models

With the emerging recognition of the role of the immune system in cancer development, the need to develop immunocompetent tumor models has become crucial. Syngeneic tumor models are established by the introduction of pancreatic cancer cells into a genetically identical host model, commonly, mouse. Moreover, both cell lines and primary cells can be used, either orthotopically or heterotopically. Notably, Panc02 and Panc03, two of the earliest cell lines purposed for syngeneic models in 1984, are derived from a 3-methylcholanthrene-induced C57BL/6 mouse pancreatic cancer model [165,205–207]. This was followed by the emergence of the metastatic site-derived, more aggressive Panc02-H7 cell line [208]. Interestingly, due to the immunocompetence of these models, high immune surveillance was detected in Panc02 tumors, and inhibition of TGF-β mediated signaling was able to reduce recruitment of regulatory T-cells, resulting in reduced tumor growth [209,210]. Furthermore, in another study by Liu *et al.* Panc02 cells were implanted heterotopically in a syngeneic model to evaluate the effect of aspirin when co-administered with gemcitabine. It was found that aspirin could potentiate gemcitabine efficacy by inhibiting the tumor-activated myeloid-derived suppressor cells and M2-polarzied macrophages [211]. However, the clinical relevance of these models is significantly limited by the lack of oncogenic *KRAS*, *CDKN2A* and *TP53* mutations which are amongst the classical features of pancreatic cancer in human in addition to a

mutation of *SMAD4* gene [202,212,213]. Also, these models do not totally represent the human PDAC TME which typically has very low immune surveillance [214].

Due to the advancement in the field of genetically modified mouse models of pancreatic cancer, many cell lines with better clinical relevance have been derived for syngeneic applications [215]. Notably, the p48-Cre-induced $Kras^{G12D}$ mutated murine pancreatic cancer model (KC) has been utilized to generate an aggressive 6606PDA (primary) and a less aggressive 6606l (liver metastasis) cell lines for syngeneic applications in C57BL/6 models [207,216]. Recently, Zhang *et al.* reported the targeting of the pancreatic cancer stroma using ECL1 (targeting peptide)-conjugated ultra-small radio-labeled and gemcitabine-loaded ^{64}Cu nanoparticles in a subcutaneously injected KPC cell syngeneic model. This nanoplatform showed favorable tumor-suppression results, reduced gemcitabine-associated toxicity as well as decreased mortality [217]. In addition to their therapeutic effect, these nanoparticles allowed the detection and imaging of the tumor tissue, proposing it as a theranostic tool in pancreatic cancer [217].

Notably, due to the similarity in the genetic background, no immune rejection develops toward the implanted tumor and therefore, immunocompetent mouse models can be used. The intact immune-tumor interaction allows for a better understanding of the immune influence on tumor growth and how different anti-cancer therapies can interact under relatively physiological conditions [187,218]. Besides the physiological immune surveillance, the complete penetrance and rapid development of tumors with high prevalence of liver metastasis and desmoplastic reaction add to the advantages of these models, however, the extent of these features is cell-dependent [203,219]. Given that the immunocompetency of these models allows for research on the tumor immune system and immunotherapy. Spear *et al.* characterized the infiltration of cytotoxic T-cells into the tumor stroma using a KPC-derived allograft model, where they were able to demonstrate the immune suppression within the tumor stroma through the analysis of various $CD4^+$ and $CD8^+$ intracellular cytokines within tumor and splenic tissues [220], however, they reported a markedly B cell infiltration in the orthotopic syngeneic model of KPC compared to the transgenic model of KPC [221]. Additionally, the availability of implantable murine cancer cells along with the lack of diversity of the genetically modified murine models, from which they are derived, are among the major limitations of this type of model [210,222].

4.2.2. Xenogeneic models

In order to better recapitulate the disease in humans, human tumor-derived cancer cells implanted in immunodeficient murine models have been commonly developed and considered as one of the major tools in preclinical oncology research, at least until recently. The development of Foxn1-mutant T-cell deficient (athymic) mice, as well as T- and B-cell deficient, severe combined immunodeficient (SCID) murine models, made it possible to introduce this type of model [223–225]. Dobrynin reported the earliest used human cell line, CaPa, which was isolated from a primary pancreatic tumor for preclinical research [226]. Additionally, Panc-1, isolated from a primary lesion, showed a high degree of stability over 2 years of culture and is one of the most commonly used cell lines in xenogeneic models [227]. Due to the clinical relevance of these models, this was followed by the development of various cell lines derived either from the primary lesion, lymph nodes or metastatic sites [210]. These cell lines have been well characterized, harboring the native human pancreatic cancer oncogenic mutations such as *SMAD4*, *CDKN2A*, *TP53* as well as *KRAS* mutations [39,228,229]. Although, classical human PDAC cell lines tend to be more chemoresponsive than their respective tumors in patients, recently, Du *et al.* have reported a novel chemoresistant human PDAC cell line (PDXPC1), which was shown to be stable and retain the original tumor features and mutations [230]. In a recent study by Prabhuraj *et al.* transferrin-conjugated, curcumin-loaded silica nanoparticles showed effective tumor inhibition results in a subcutaneous Mia PaCa-2 pancreatic cancer model. Additionally, co-treatment with gemcitabine showed synergistic effects, which reflects the chemoresistance inhibitory effect of the nanoformulation [231].

A range of pancreatic cancer-associated oncomiRs have also been identified within these cell lines via transcriptomic analysis, controlling tumor-driving genes such as *CD40*, *RAC1*, *TP53* and *BCL2* [202,232–235]. However, due to the cell lines' tendency to mutate, their clinical relevance was reported to be highly liable and classified to be of moderate value by the National Cancer Institute [236]. Moreover, their therapeutic response is reported to be inconsistent [237–240]. This might be explained by their ability to adapt through constant passaging in *in vitro* culturing conditions, and select the most proliferative and resistant variants to survive. As a result, these cell lines have limited capacity to represent the real tumor of origin [39,236–238,241,242]. Additionally, significant variations in proteomics are observed between the cultured cell lines and the patients tumors [202]. Along with the genetic variations of the cell lines, they represent a limited population of the pancreatic cancer patients. These human cell lines possess varying tumorigenic potential and differences in metastasis, chemoresistance or ability to recruit stroma [243,244]. Furthermore, the lack of immune response and proper tumor stroma in host animals adds up to the limitations of these models, where they fail to simulate the potential pro- and anti-tumoral influences of stromal and immune cells, making them unsuitable for therapies targeting the TME components [213,245].

4.2.2.1. Heterotopic injection. Among the majority of heterotopic models, subcutaneous injection of human tumor cells in the flank region of the murine model is the most favored due to convenience, ease of establishment, time and cost effectiveness. This model allows the visual tracking of tumor vasculature, growth, and therapeutic response by means of tumor size [246,247]. After cell implantation, tumors can be palpated within an average of 4 weeks, depending on the cell line aggressiveness and rate of proliferation [248]. This model is suitable for the co-injection of human tumor cells with fibroblasts or PSCs in order to mimic the real human tumor stroma [249,250]. However, the clinical relevance of subcutaneous models is highly affected by the lack of metastasis, which is a common feature of PDAC in humans. Moreover, subcutaneous tumors are surrounded by a capsule-like membrane and supplied by skin-derived vasculature, which is distinct from the pancreatic blood supply network compared to respective orthotopic tumors [202,210].

Nevertheless, some interesting biology can be observed in these models. D'Aronzo *et al.* implanted subcutaneous tumors in athymic mice and showed that fasting potentiates the efficacy of gemcitabine via upregulation of the hENT1 transported under starvation conditions [251]. Another study used subcutaneous tumor models of Panc-1 and Mia PaCa-2 cells to demonstrate the role of BRG1 in gemcitabine chemoresistance [252]. However, in the study by Oon *et al.*, an inconsistency between *in vitro* studies and *in vivo* Panc-1 xenograft tumors was reported. They showed that EX527, a SIRT1 inhibitor, was able to inhibit chemoresistance *in vitro*, however, this was not observed *in vivo* [245,253], which was attributed to the potential role of the TME *in vivo*.

Due to the poor stroma recruiting potential of human cancer cells when injected into murine models, Kuninty *et al.* have reported the co-injection of human PDAC PANC-1 cells and PSCs subcutaneously in the SCID murine model [33]. The established (PANC-1 + PSC) tumors demonstrated a high degree of desmo-

plasia, with high expression of the CAF marker, α-SMA and increased collagen levels when compared to PANC-1 alone. Besides the biological relevance, therapeutic testing of a novel peptide (AV3) targeting integrin alpha 5 receptor overexpressed on CAFs, with or without gemcitabine, showed significant therapeutic effects in co-injection model, which were consistent with *in vitro* data in 3D co-culture spheroids as well as in a patient-derived xenograft model (PDX). Another desmoplasia mimicking model was reported by the co-injection of human PDAC cells SUIT-2 and PSCs. In a similar fashion, the expression of α-SMA was elevated in the co-injection model compared to the tumor cell only model, and the efficacy of the stroma-targeting agent, pirfenidone, was reported [254]. Similarly, Masamune reported the PSC-dependent growth inhibitory effect of Olmesartan in an AsPC-1 co-injection model [255].

4.2.2.2. Orthotopic injection. Although it is a sophisticated and labor-intensive model, tumor implantation into pancreatic tissue significantly improves the biological relevance of the model when compared to the subcutaneous tumor implantation [256–258]. Orthotopic models are favored due to their uniform growth kinetics, tendency to metastasize, and more representative drug penetration [202,259,260]. Infiltration of pancreas-derived stromal components helps to mimic PDAC in humans by introduction of the highly dense TME, which is among the key features of PDAC.

Tumor implantation is usually performed in the tail or head of the pancreas. Pancreatic head-implanted tumors are highly liable to invade into the pancreatic tissue as well as developing severe liver and biliary duct metastasis, which is in line with human PDAC manifestation. However, in the case of pancreatic tail-implantation, slower tumor growth is reported and metastasis occurs more frequently [210]. In addition to cell line selection and number of injected cells, the technique of implantation plays a major role in determining the tumor growth pattern [210]. The simplest method is carried out by direct injection of 2D-derived culture tumor cells into the tail of the pancreas after incision under anesthesia, maintaining the sterility. Another more complicated method is implemented by the subcutaneous implantation of tumor, followed by the collection of the established tumor tissue. The tumor can be either cut into 1–2 mm fragments and implanted in the pancreas in a homograft fashion or disintegrated as a cell suspension and implanted into the pancreas as previously described with 2D-derived cells [261–263]. Tumor cell suspension implantation results in a higher incidence of metastasis, compared to tumor fragment method, hence, is often used in metastasis-related studies [210,262].

Unlike subcutaneous models, one of the critical challenges in orthotopic models is real-time tumor growth tracking. However, ultrasonography imaging, bioluminescent imaging of luciferase-expressing cell lines and the implantation of a dorsal window chamber have been implemented by the researchers to track murine orthotopic tumor growth [264]. Moreover, susceptibility to infections can add to the limitations of these immunodeficient murine models in xenogeneic models if researchers do not imply adequate sterile techniques [202]. In addition, the diffuse nature of murine pancreatic tissue makes tumor injection process challenging and highly prone to inconsistency and reproducibility of tumors. Cell leakage from the injection site is among the drawbacks of these models as it can lead to the dissemination of peritoneal spread, which can be minimized by embedding the cells in a viscous vehicle such as Matrigel [265]. Besides that, the time consuming nature of this orthotopic injection surgery requires the preparation of cells at multiple time points in order to avoid the injection of exhausted cells due to the long standing time, which can lead also to the inconsistency of tumors.

Likewise in subcutaneous implantation, desmoplasia can be recapitulated in orthotopic models by either injection of stroma-recruiting tumor cells or the co-injection of tumor cells and CAF-progenitor cells such as PSCs. Host stroma recruitment is limited by the expression of specific cytokines and growth factors by tumor cells. A previous study has shown that an orthotopic model of TGF-β1-transfected PANC-1 cells demonstrated a marked CAF recruitment to the stroma, emphasizing the role of TGF-β1 [266]. In another orthotopic model, Bailey *et al.* reported the induction of stroma by the injection of Capan-1 cells into the pancreas, where treatment with Sonic hedgehog (SHH) inhibited it [267]. Co-injection of tumor cells and PSCs has also been reported. Vonlaufen *et al.* demonstrated the incremental effect of co-injection of MiaPaCa-2 and PSCs on tumor growth as well as on the incidence of metastasis, compared to the implantation of only tumor cells [268]. In concordance, another study reported that the incidence of metastases was found to be proportional with the ratio of PSCs/BxPC-3 cells injected in the pancreas [269]. Moreover, in order to model different PDAC subtypes, a recent work by Miyabayashi *et al.* has investigated the intraductal implantation of single-cell suspension of human-derived tumor organoids into murine pancreas. Via this technique, they were able to generate two distinct subtypes of ductal lesions with high engraftment rates compared to the traditional orthotopic implantation. Notably, one of the phenotypes is characterized by its slow development and less desmoplastic nature, while the other subtype is fast-developing and invasive with predominant desmoplasia [270,271].

4.2.3. Implantation of patient-derived xenograft (PDX) tissues

Patient-derived xenografting is one of the most advanced techniques implemented in preclinical tumor studies and a means of testing patients' samples *in vivo* using a personalized medicine approach. These represent a subtype of xenogeneic tumor implantation in immunocompromised mice, where the implanted tumor is derived directly from fresh human tumor tissues instead of *in vitro* derived cultures. Additionally, PDX models have been reported, which are established from metastatic sites and circulating tumor cells extracted from fresh patient blood [272]. Grafted human tumors are usually fragments of around 1 mm^3 in volume, however, subcutaneous implantation can be performed, for propagation of the primary xenograft, followed by collection of the tumor and re-implantation [273]. Immunodeficient mice strains, including T-cell deficient nude mice (athymic), T-, B- and NK-cell deficient NOD-SCID mice and SCID-beige mice, are used as hosts for PDX tissues [274]. In order to develop a clinically relevant immunocompetent mouse model, mutation in the IL-2 receptor gamma common chain was introduced in NOD-SCID-based model (NSG). The severe immune impairment due to the loss of functionality in T-, B- and NK- cells as well as the IL-2 receptor mutation allowed for the inoculation of human immune progenitor cells such as hematopoietic stem cells (precursors of hematopoietic-derived immune cells) and peripheral blood mononuclear cells (precursors of human white blood cells, especially T-cells) which in turn add to the clinical relevance by mimicking the human immune system, hence, denoted as humanized murine model [202,275–277]. Humanized mice are divided into 3 strains; BRG (BALB/c $Rag2^{-/-}$ IL-2Rγc$^{-/-}$), NSG (NOD.Cg-$Prkdc^{scid}$ $Il2r\gamma^{tm1Wjl}$) and NOG (NOD.Cg-$Prkdc^{scid}$ $Il2r\gamma^{tm1Sug}$) [275,278]. In a more recent study, Miao and colleagues developed an Il2rγ-knock out Syrian hamster model for PDX application [279]. Interestingly, their results demonstrated high metastasis rates, desmoplasia and EMT changes after orthotopic and subcutaneous implantation of xenografts, proposing this model as a superior host for PDX compared to immunodeficient mice [279].

PDX models address the limitations of the cell-line-derived xenograft models including the mutation and selection of cancer

cells. PDX also mimics the human tumor by the incorporation of the heterogenous components of real human PDAC tissue, including the stroma, which aids in increasing metastasis and chemoresistance [280–283]. PDX models are capable of preserving the histopathological features of the primary patient-derived tumor including the vasculature, lymphatic drainage and necrosis [284]. Subcutaneous implantation is the most commonly used method due to the high success rate and ease of monitoring, however, orthotopic PDX models demonstrated better biological relevance and increased metastasis but are more challenging to monitor [244,258,285,286]. Renal capsule implantation has also been reported as a modality to improve the implantation success rate [287].

PDX tumors can be maintained and expanded by passaging into a series of murine hosts. However, human stromal cells are reported to be retained for 3–5 and up to 10 generations in a size and time dependent manner but subsequently diminished and replaced by murine stromal cells during the course of tumor growth and are not detectable in later passages [273,288–291]. Furthermore, genetic deviations can occur due to expanded cycling, however, key genetic mutations were reported to be highly stable for up to 39 generations [292]. Additionally, one of the important strengths of PDX models is the ability to test therapeutic agents in individual patient samples as a means of personalized medicine, which gives an estimation of the extent of response to certain therapies despite the lengthy testing procedure [210,244]. With regards to the slow growth rate of PDX tumors, the lag time between implantation and tumor development is 2.5 months on average which may limit the personalized medicine application in most cases. The success rate of tumor implantation reaches an average of 50% of cases and is dependent on several factors, including tumor size, ratio of stroma cells, site of implantation, mouse strain, extent of necrosis, lymphatic drainage, tumor vascularity, and the extent of the tumor cells viability [245,258,293,294]. Notably, failure of PDX implantation positively correlates with positive tumor-bearing patient prognosis [295,296].

In a study conducted by Zhou and colleagues on an orthotopic PDX model, insulin-like growth factor-1 (IGF-1) was conjugated to doxorubicin-loaded iron oxide nanoparticles as a targeting ligand to IGF-1 receptor, which is overexpressed in PDAC tumor cells as well as the surrounding stromal cells [297]. Their results demonstrated a superior anti-cancer effect in the IGF-1-conjugated nanoformulation compared to the free drug group [297]. In another NSG PDX model, Witkiewicz and colleagues successfully inhibited pancreatic tumor growth using a combination of an Akt inhibitor (dasatinib) and MEK inhibitor (trametinib), confirming the clinical relevance by overcoming the known compensatory Akt mechanism to MEK inhibition in PDAC [291,298,299]. More recently, Huang *et al.* used a pancreatic cancer chemoresistant PDX model to evaluate the synergistic efficacy of a nanoparticle-loaded cyclin-dependent kinase 7 inhibitor (THZ1) and bromodomain-containing protein 4 inhibitor (JQ1) [300]. They demonstrated the augmented effect of the combined treatment on tumor inhibition as well as reduced liver toxicities associated with the used drugs compared with free drug forms [300]. With all progress seen in the field of PDX pancreatic models to translate the pre-clinical screening, it remains to be seen from clinical trials how effective these approaches are in patients at which it is not possible to fully assess the predictive power of these models in preclinical settings.

Due to the highly dense TME, radiographic imaging of PDAC in humans has been challenging, suggesting PDX as a suitable model for development of diagnostic and imaging techniques. Given the high expression of epidermal growth factor receptor (EGFR) in human PDAC tissue, Boyle and colleagues demonstrated that 64Cu-labelled anti-EGFR monoclonal antibody fragments selectively bound to orthotopic and subcutaneous PDX tumor tissue, which can then be visualized using microPET/CT imaging. This was proposed as a promising imaging modality in human PDAC [301]. Drawbacks associated with this model include the heterogeneity in growth rates among different specimens. Moreover, in some cases, xenograft specimens may have insufficient living tumor cells to be implanted. Furthermore, specialized training is required for tumor harvesting, viability preservation and implantation [210]. Overall, clinical trials are yet the most effective step in validating these models via therapeutic testing.

4.3. Genetic mouse models

Despite the reasonable clinical relevance of some of the cell- and tissue-based pancreatic cancer models, genetically engineered mouse models (GEMMs) have been shown to give a better understanding and more realistic recapitulation of human pancreatic cancer on a number of levels, namely its rapid development, desmoplastic nature, aggressiveness, early invasive spreading to lymph nodes and distant organs as well as associated cachexia and ascites [245]. In addition, GEMMs carry the advantage of being hosts suitable for immunotherapeutic studies due to their intact immune functions. Due to mixed genetic mutations associated with human PDAC, researchers have investigated the roles of several of these to further elucidate mechanisms of cancer progression and to develop therapeutic approaches. Notably, the presence of early key oncogenic drivers in these models such as *Kras*^{G12D} mutations as well as late events such as *Trp53* mutations allowed for typical simulation of pancreatic carcinogenesis in humans through primary tumor lesions including pancreatic mucinous cystic neoplasm, intraductal papillary mucinous neoplasm (IPMN) as well as the most common form, pancreatic intraepithelial neoplasia (PanIN) [302,303]. Additional genetic ablations of the tumor suppressors Smad4 and p16^{Ink4a} demonstrated a better recapitulation of the invasive phenotypes of PDAC [304,305]. Notably, single mutation models are not capable of simulating the complete picture of PDAC tumorigenesis in a timescale conducive to therapeutic testing, therefore a compound oncogene model is preferred. However, in contrast to the high number of genetic mutations required for PDAC initiation in human, as low as two or three genetic mutations are required to form malignant transformation of the pancreas in murine models [306,307].

Researchers have developed GEMMs via introduction of transgenes, or conditional gene manipulation (either deletion, mutation or activation) using, for example, Cre-Lox technology. Transgenic technique comprises tissue and stage non-specific gene transfer to the murine genome. However, in the most commonly used models, transgenes incorporating tissue-specific promoters fused to Cre recombinase are used in order to achieve selective targeting of genetic lesions to the pancreatic epithelium [308]. Researchers have recognized several important promoters that act during the course of pancreatic development and homeostasis including homo-domain transcription factors such as pancreatic and duodenal homeobox 1 (Pdx1), NK homeobox family 6-1 (NKX6-1) and motor neuron and pancreas homeobox 1 (Mnx1) as well as neurogenin 3 (Ngn3), basic helix-loop-helix transcription factor p48/-pancreas transcription factor 1 (p48/Ptf1) complex, and elastase (Ela) [200,302,303,308–314]. Due to their importance in murine pancreatic development and due to their relative specificity to the pancreatic epithelium, *Pdx1* and *p48/Ptf1* are the most commonly used promoters to drive Cre which can then control the recombination of conditional alleles to mimic mutations and deletions seen in human PDAC. However, in contrast to human PDAC, expression of pancreatic oncogenes/loss of tumor suppressor function is initiated in the premature embryonic progenitor cells of the GEMMs rather than in the fully differentiated adult pancreas as in

human, which may affect how well PDAC in murine models reflects the human disease [307]. Furthermore, despite the high ability to recapitulate the clinical features of PDAC in murine models as well as relatively short latency of tumor development, the simultaneous activation of target oncogenes with inactivation of tumor suppressor genes doesn't reflect the naturally-occurring sequential deregulation of these genes in PDAC [204,315]. Some of these drawbacks can be tackled using more advanced conditional strategies.

For example, conditional technology allows for an accurate control of gene manipulation at specific locus and specific stage of development. Tet-on and Cre/loxp recombinase systems are the most widely used techniques, while LSL (floxed STOP transcriptional cassette) is commonly used for gene knock-in procedures to allow expression of site-specific mutated genes from their endogenous loci [316–318]. As it is considered the key driver in PDAC initiation, emerging of *Kras*^{G12D} mutant mice was a milestone in GEMM development [311,319]. Initially, the KC model, (*LSL-Kras*$^{G12D/+}$; *Pdx1-Cre*) was developed via the interbreeding of *LSL-Kras*^{G12D} mice and pancreas-specific Cre-recombinase-expressing mice where Cre expression was maintained by pancreas-specific Pdx1 promoter [302]. The expression of mutant *KRAS*^{G12D} within the pancreatic tissue drives PanIN initiation in the early weeks of the animals' life, which slowly develops and transforms into a more aggressive lesion and ultimately, metastatic pancreatic cancer, albeit at a low frequency and long latency [302,320]. The reported PanIN lesions were found to mirror the histopathological features of human precursor lesions [18]. However, more research has been implemented to generate more advanced models with compound genetic mutations to overcome KC drawbacks such as latency and incomplete penetrance. The concurrent activation of proto- oncogenes and inactivation of tumor suppressor genes has been viewed as an effective strategy to develop GEMMs that recapitulate human PDAC successfully with a shorter latency and more complete penetrance [204].

In order to test TME-targeting therapeutic strategies, several PDAC-mimicking desmoplasia-rich models have been developed. Further manipulations of the KC models have been performed via conditional incorporation of the *Trp53*^{R172H} mutant, which is known to occur in the majority of human PDAC cases, into KC models [321]. Namely, KPC (*LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx1-Cre*) mice, one of the most commonly used GEMMs in mimicking human PDAC, were generated by crossing in the LSL-Trp53^{R172H} allele [303]. This compound mutation resulted in the development of spontaneous, highly desmoplastic and rapid-developing PDAC with latency of less than 6 months [303,322]. Notably, the mice demonstrated the complete spectrum of precursor lesions at an average age of 9 weeks, followed by the fully developed and invasive tumors 8 weeks later. In addition, the mice exhibited many of the features of human PDAC including weight loss, abdominal enlargement and high metastatic dissemination mainly to lungs and liver as well as diaphragm and the adrenal glands [323]. Moreover, in common with human PDAC, Provenzano and colleagues reported tumors in KPC mice to have excessive deposition of ECM proteins, especially hyaluronic acid, glycosaminoglycans and collagen [203,324]. In-depth differences between KPC and KC models have been highlighted by Ariston Gabril [325]. In addition, other tumor suppressor pathways have been investigated in models based on KC mice. *Tgfbr2* deletion was shown to be capable of inducing pancreatic desmoplasia in the presence of *Kras*^{G12D} mutation [326]. Another stroma-inducing strategy was reported by the activation of Snail under the control of an Elastase-driven tetracycline-dependent conditional model, which showed excessive collagen deposition in the TME as a result of TGF-β activation [327]. Another tetracycline-dependent Elastase control system was reported by Krantz and colleagues, where membrane type-1 matrix metalloproteinase (MT1-MMP) overexpression in the pancreatic TME disseminated IPMN and excessive fibrotic stroma as a result of TGF-β and SMAD2 overexpression [203,328].

Pten deletion has been shown by several groups to accelerate tumor development in KC mice [12,329], whilst in an effort to develop a cachectic model for therapeutic research, Talbert and colleagues, generated an inducible *LSL-KRAS*^{G12D}; Pten$^{f/f}$ model under the control of tamoxifen-inducible Ptf1a-Cre recombinase, named KPP [330]. Another model of tumor suppressor inactivation was generated by the conditional deletion of cyclin-dependent kinase inhibitor 2A (*Cdkn2a*), the gene that encodes both p16^{Ink4a} and p19^{Arf}. Loss of Cdkn2a and activation of *KRAS*G12D under the influence of Pdx1-Cre resulted in the emergence of a rapid developing and invasive model, so-called, KIC (*LSL-KRAS*^{G12D}; *Ink4a/Arf*$^{lox/lox}$; *Pdx1-Cre*) [311,312,331,332]. This model demonstrated rapid PanIN lesion development compared to the KC model, with shorter life expectancy (8 weeks vs 1.5 years) as well as more invasive characteristics [10,302,311]. Further tumor suppressor mutated models, in the context of *KRAS*^{G12D} mutation, include SMAD4 inactivation, denoted as the KD model, which showed early onset of IPMN and a moderately aggressive tumor phenotype [323]. Interestingly, Schönhuber *et al.* were able to combine both Cre-loxP- and flippase Flp-*FRT* recombinase systems into a dual recombinase system. This strategy enabled the researchers to investigate the tumor development in a stepwise manner. Moreover, via this technique they were able to selectively target tumor tissue and stroma [333]. In another study by Guerra *et al.* PanIN and subsequently PDAC can develop from *KRAS* oncogene-harboring acinar cells under prolonged inflammation. This transformation to PDAC can be arrested if tissue repair is initiated and anti-inflammatory treatments are given [320].

Therapeutic testing on GEMM pancreatic cancer models has been an important step towards drug development in preclinical studies. In a study reported by Ocal and colleagues, they traced the therapeutic efficacy of different molecules in a KIC-crossed RGS16:GFP bacterial artificial chromosome (BAC) transgenic model by quantifying the level of *KRAS*^{G12D} expression throughout the treatment. Rgs16::GFP was reported to be a *KRAS*-dependent tumor reporter, which correlates with tumor burden. The tumor-suppressive effects of gemcitabine and receptor tyrosine kinase Axl inhibitors were observed by means of reduction of GFP expression in the tumor tissue. This *in vivo* model provides a highly sensitive *in vivo* screening tool of tumor inhibitory molecules and chemotherapy, allowing follow up of tumor burden via the Rgs16::GFP reporter [334].

Moreover, due to the desmoplastic nature of the KPC model, TME-depleting strategies have been tested. Olive and colleagues used the KPC model to observe the SHH signaling inhibition using IPI-926. Inhibition of desmoplasia was observed, which resulted in improved drug delivery and augmented effects of gemcitabine [335]. This was supported by the chemoresistance-inducing effects of CAFs in a study reported by Hessmann and colleagues using the KPC model [336]. Furthermore, in a KPC-based model, PEGPH2 was shown to reduce hyaluronic acid deposition in the TME, and subsequently, augmented the effects of gemcitabine and reduced mortality [324]. KPC mice were also utilized to test the anti-fibrotic effect of an αvβ3 integrin-targeting therapy, which resulted in CAF-depletion as well as reduced chemoresistance [337]. It is worth noting, however, that clinical trials of these concepts have failed, and there is now significant effort to refine stromal targeting therapies based on specific tumor-promoting pathways in the stroma, rather than complete ablation.

Of importance, inactivation of the *KRAS* oncogene has shown positive effects as an anti-tumor strategy, although due to the

infeasibility to develop direct molecular inhibitors, except in the case of *KRAS*^{G12C}, targeting its downstream effectors has been of high interest [338–342]. However, in a recent study, Strand and colleagues were able to arrest pancreatic tumor growth in the KPC model via targeted delivery of *KRAS* gene-silencing (siRNA)-loaded nanoparticles to tumor cells [343]. Given the immunocompetence advantage of GEMMs, suppression of PDL1 and IL-20 in KPC model showed promising effects in tumor growth inhibition and relief of tumor-associated cachexia [344]. Jiang *et al.* identified the overexpression of focal adhesion kinase (FAK) to be associated with poor PDAC stroma penetration of killer CD8$^+$ T cells. Via inhibition of FAK using FAK inhibitor VS-4718, they were able to increase the survival in KPC model. Inhibition of this enzyme lead to reduction in immunosuppressive stromal cells infiltration as well as enhanced sensitivity towards the PD-1 immunotherapy [345]. Use of CD40 agonist was found in a study by Beatty *et al.* to significantly reverse tumor stroma-induced immunosuppression. This tumoricidal effect was shown to be initiated by CD40-activated macrophages to a superior extent to T-cells or gemcitabine treatment [346].

Current advances in PDAC animal modeling have been able to mimic a reasonable part of human PDAC tumors, however, none of them has been accepted as a standard, which meets all PDAC features. Despite the enormous advances in the understanding of PDAC biology and the development of highly sophisticated PDAC-recapitulating animal models for translational research, these models have failed to faithfully translate into clinically-validated models. Genomic analysis of PDAC patients proposed 12 different root signaling pathways involved in PDAC pathogenesis, from which only a narrow spectrum has been investigated in GEMM models [347,348]. More research needs to be focused on extensively exploring the whole set of pathways as well as new biomarkers of PDAC from a different prospective, which may grant a better understanding and emergence of more clinically-relevant animal models.

5. Conclusions & future challenges

PDAC is one of the most lethal types of cancer mainly due to the usually late diagnosis, complex environment, high treatment resistance and aggressive nature. In particular, the TME plays a significant role in the progression but also in the resistance of PDAC towards conventional therapeutic strategies such as chemotherapy, which started a completely new line of research that is more focused on the targeting of the TME components rather than the tumor cells themselves [7,23,349–352]. Recent studies describing the identification of novel TME components such as iCAFs or ap-CAFs demonstrate that the PDAC TME is not yet fully understood. Novel interactions that might display a crucial effect on drug efficacy and performance are yet to be discovered. Different models, *in vitro* as well as *in vivo*, can aid in further understanding the complex interactions within the PDAC TME as well as serve as suitable and biologically relevant platforms for drug development and evaluation. In the last few years, several platforms have been developed that are able to mimic the *in vivo*-like situation, ranging from simple cell-based platforms to study specific interactions within or between different cell types to complex patient-derived models that are able to mimic tumorigenesis and the complex TME to a greater extent. However, it is important to highlight that a more complex model does not necessarily mean a better model. Eventually, every model should be suited to its final application which means both *in vitro* and *in vivo* models are crucial for the development of novel therapeutics that aim to treat PDAC.

5.1. Major developments in 3D in vitro and in vivo models

Over the recent years, the developments in the field of PDAC *in vitro* models have been impressive ranging from simple 3D spheroids towards complex 3D bioprinted or PDAC-on-chip systems. In particular, the focus on the proper implementation of the PDAC TME to create biologically relevant tumor mimics makes the application of these models highly interesting for the development and evaluation of novel PDAC therapeutics in a high-throughput and cost-effective manner. Furthermore, the aforementioned *in vitro* models are continuously and rapidly improving in terms of biological relevance based on new developments in biomaterials, engineering or cell culture methods. For instance, novel developments in bioprinting focus on avoiding a scaffold material to print the cells, instead relying on the cells own ECM production [353]. Another reason for the rapid growth of the 3D *in vitro* model field is the combination of multiple disciplines while developing novel models. Current developments show the benefits of, for example, combined organoid with organ-on-chip technologies to create a patient relevant tissue in a confined and well-controlled environment or combined bioprinting with organoid and spheroid technologies to increase the reproducibility of the cultures [85,354]. The combination of different technologies allows for the development of 3D *in vitro* models that are able to replicate the architecture and composition found in human PDAC to a great extent, and as cells from patients can be directly used in such models, allows for the comparatively fast and cost-effective set up of patient-relevant *in vitro* models, paving the way for the application of such models in personalized medicine.

Similarly, animal models have developed from simple injection models towards platforms that are able to mimic tumor development based on genetic alteration or chemical inductions similar to the situation in patients. *In vivo* models have developed tremendously from the poorly patient PDAC-mimicking chemically induced models to the highly sophisticated genetically engineered mice models and (humanized) PDX models.

5.2. Remaining challenges

Despite several advantages of animal models, a lot of focus has recently been given to 3D *in vitro* models aiming to replace animal studies. Although this would be admirable from an ethical point of view, as previously stated, *in vitro* models do not yet present the complexity to fully replicate the situation in PDAC patients. Especially in drug development, this complexity can greatly alter drug efficacy and render different drugs useless. Although recent developments in *in vitro* models try to implement the stromal barrier found in PDAC, include immune components, and try to connect different organs in a multi-organ approach, such systems are still juvenile and do not yet meet the requirements to fully replace animal studies. In particular, the integration of the vascular characteristics of PDAC such as compressed blood vessels and a reduced EPR effect are so far missing in current 3D *in vitro* systems. Arguably, 3D bioprinting and organ-on-chip systems would present the most suitable platforms to integrate a controlled vasculature due to the versatility and high control these platforms offer, however, so far no vascularized PDAC platform has been reported to integrate these characteristics. Although an EPR effect could recently be simulated in an organ-on-chip platform, by using TNFα to initiate leakiness in a monolayer of HUVECs, so far no model covers the collapse of vasculature in PDAC *in vitro* models, which forms a crucial characteristic for drug resistance [355]. The lack of proper vascularization in current 3D *in vitro* models also limits the presence of another PDAC characteristic in 3D *in vitro* models – the highly

dense ECM network consisting of, among others, high levels of collagens, hyaluronic acid and fibronectin as previously described. The implementation of such high levels of ECM in current *in vitro* models would only be feasible with increased vascularization of models as otherwise cells would lack the necessary nutrients and oxygen supply as extensively discussed elsewhere [356,357]. Although spheroids often claim a high density based on the presence of cell-produced ECM, the levels and density of this ECM do not entirely match with stroma present in PDAC patients. Moreover models that are based on scaffolds, including 3D bioprinted models or OOC platforms, as well as models using scaffolds such as organoid cultures, often use hydrogels that are significantly softer than the actual ECM found in PDAC [358]. As the ECM stiffness does not only present a direct physical barrier towards chemotherapy but can also alter the behavior of for instance cancer cells [359], matching the stiffness and density of the ECM in 3D *in vitro* models with the actual ECM found in PDAC patients is a crucial steps towards physiologically relevant 3D *in vitro* PDAC models.

A different challenge that arises while increasing the complexity of 3D *in vitro* models to better match the situation *in vivo*, is to still allow for a simple and effective read-out. Arguably with each additional cell type and ECM component, the read-out of the model becomes increasingly challenging depending on the type of model and aim of the final application. While an increasing complexity of the model can also be beneficial when studying for instance the penetration of drugs, where a high complexity better represents the realistic situation while the general read-out (confocal microscopy or tissue sections) remains the same, when studying specific cell–cell, cell-ECM or cell-drug interactions an increased model complexity directly complicates a clear read-out. Depending on the analysis method, different challenges can arise: For instance, confocal microscopy is widely used in the analysis of 3D *in vitro* tissues, however, is often limited to a penetration depth of 100 μm depending on the tissue density and overall tissue structure [360,361]. While this depth would still be sufficient to image small spheroids, organoids, or PDAC-on-chip tissues, larger objects including scaffold-based tissues or larger spheroids cannot be imaged entirely. Multiphoton microscopy has recently demonstrated higher penetration depths compared to 2-photon-microscopy, which depending on the tissue can solve the penetration depth issue. Recently, however, a different promising approach emerged to image larger-scale tissues based on the full clearing of tissues using for instance solvent-based approaches [362–364]. Clearing the whole tissues significantly increases the penetration depths and allows for imaging larger-scale *in vitro* or even *in vivo* tissues. A crucial aspect about microscopic imaging of 3D *in vitro* tissues in general is the proper labeling of the cells or components of interest. Although cells can be in general labelled using antibodies of interest, highly dense models might prevent the antibody penetration and therefore do not give an adequate and reliable read-out. To overcome this issues, cells can be labelled individually before generating the 3D models using commercially available dyes (CellTracker™, QTracker™ and others [365]) or by transfecting cells to stability express a certain fluorescent protein such as GFP [366]. Although these approaches allow to individually track cells in microscopy, it has to be taken into account that commercial dyes often loose fluorescence based on increased cell division, while a stable transfection of cells might be time-consuming and challenging depending on the cell type [365]. Similar problems related to the proper labelling of cells also arise when analyzing cells using for instance flow cytometry, including fluorescence-activated cell sorting (FACS), or while analyzing the gene expression. Although cells are usually sorted by specific surface markers using FACS, and can then be individually analyzed for their gene or protein expression using polymerase-chain reaction or Western Blot [136,367], not all cell types express specific markers on their surface that allow for proper cell sorting, often leaving cell labelling with commercial dyes or transfection the only reliable option. In general, the analysis and read-out depends on the final aim of the study. The more complex the model, the number of cells and ECM involved and how specific the final analysis will be, the more challenges have to be faced in the read-out of the model. This might also include the design and development of completely novel assays fitting the specific 3D *in vitro* model. Nonetheless, 3D *in vitro* models still offer several methods to allow for a simple read-out such as the pre-labelling of cells with dyes, the high control of the size as well as the control on the architecture and the complexity of the model, which cannot be performed or controlled *in vivo*.

Besides these technological limitations, a different challenge that remains is convincing researchers that are primarily in the field of drug development to use novel *in vitro* models for the drug evaluations. In most laboratories that focus on the development of novel therapeutics, the evaluation pipeline starting from initial evaluation in 2D followed by animal models is already set up and optimized. The inclusion of additional 3D *in vitro* models might be seen as an unnecessary and costly addition to the working evaluation pipeline and might require additional expertise to set up, especially in more complex models such as organoid, bioprinting or PDAC-on-chip models. As a result, the use of 3D *in vitro* models, although ethically admirable, might be challenging.

5.3. Towards an efficient drug development pipeline

Based on these technological and psychological challenges that *in vitro* models face at the moment, it is too early to convincingly see *in vitro* models as potential replacements for *in vivo* models. However, 3D *in vitro* models should be included in the evaluation pipeline to aid in the proper evaluation of novel therapeutics, which might eventually reduce the number of compounds failing in pre-clinical and clinical stages of the development phase. Such models can be used to further optimize therapeutics in a more relevant environment compared to conventional 2D cultures before embarking on animal studies. In this way, only the most promising candidates will be tested in animals eventually reducing the number of animals necessary. So far only a few studies have involved both, *in vitro* and *in vivo* models, in a single development pipeline, however, as shown by Schnittert, Heinrich *et al.* and Kuninty *et al.*, 3D *in vitro* models can predict the efficacy and performance of a novel therapeutic *in vivo* [33,77]. Both studies involved evaluation of a novel compound in 3D heteropsheroids consisting of pancreatic cancer cells and PSCs before embarking on animal. In these studies spheroids and *in vivo* models displayed a highly similar inhibition profile after drug treatment, demonstrating how 3D *in vitro* and *in vivo* models correlate (Fig. 4). In such a way, 3D *in vitro* models do not replace animal studies but reduce animal studies, rendering the eventual studies more efficient and cost-effective, which can alter the way for faster clinical studies as time and budget can be shifted towards clinical phases.

5.4. Role of computational simulations and artificial intelligence (AI)

Besides *in vitro* and *in vivo* models, recently a lot of focus has also been given to the development of computational "*in silico*" models based on different artificial intelligence and machine learning techniques. In the last few years, *in silico* models have been used for a variety of cancer-related applications including the simulation of cellular processes within cancer, invasion and progression of different cancer types, studying the interaction of different TME components based on specific pathways as well as simulating immune response towards cancer cells [368–372].

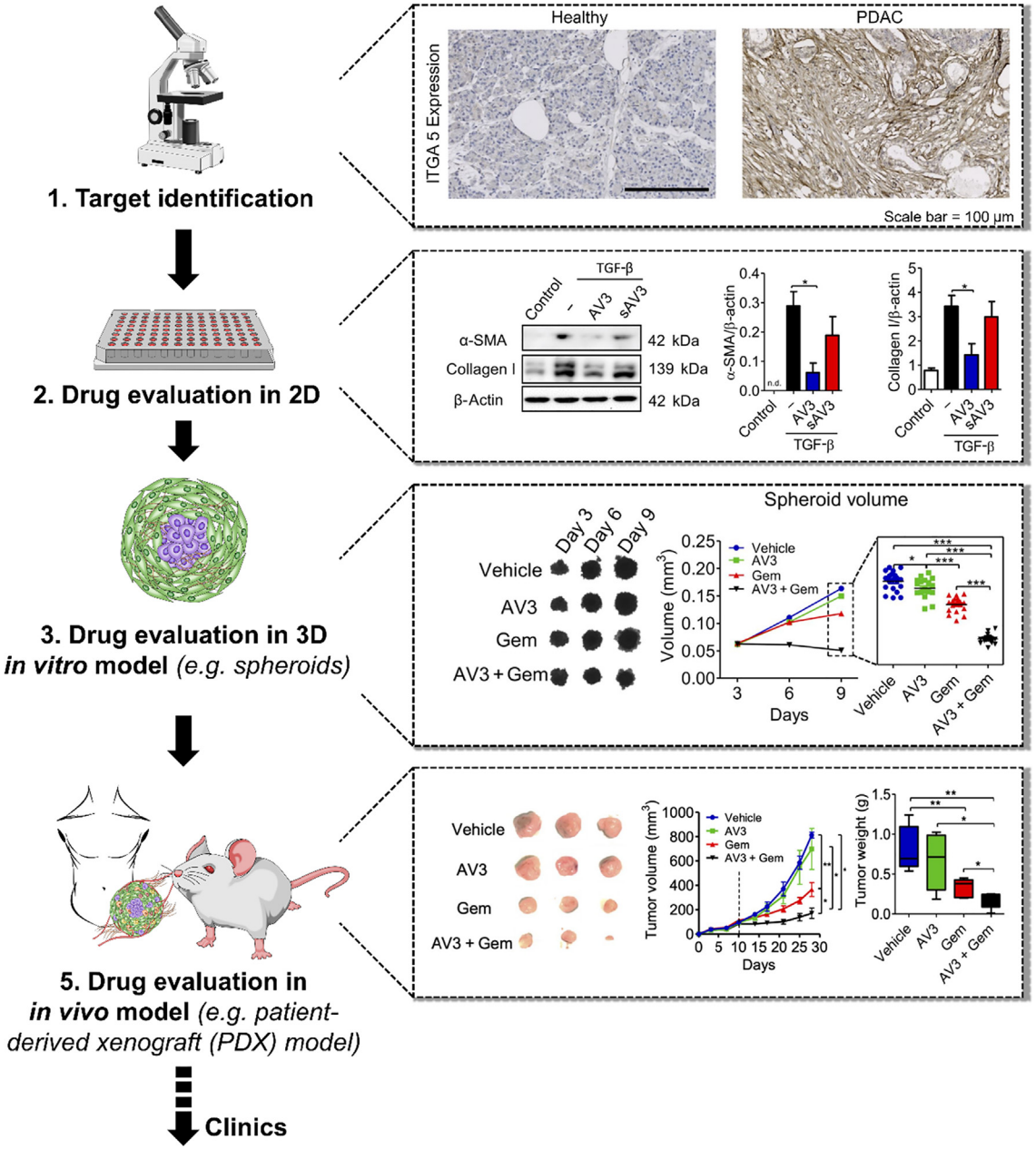

Fig. 4. Overview of a possible drug development pipeline highlighting published data for each step (Kuninty *et al.* [33], published with permission under the CC-BY-NC license. Copyright © 2019 The Authors). (From top to bottom) 1. Target identification represented by an immunohistochemical staining of integrin a5 (ITGA5) in pancreatic tissues of healthy and PDAC patients. 2. Initial drug evaluation in 2D represented by Western Blot of AV3 (ITGA5 antagonist) and silenced AV3 (sAV3) demonstrating the efficacy of AV3 to inhibit PSCs. 3. Drug evaluation in 3D PSC-Panc1 heterospheroid model demonstrating the efficacy of AV3 with and without the co-treatment of gemcitabine (highlighting the spheroids size, growth over time and volume on day 9). 4. Drug evaluation *in vivo* in patient-derived xenograft (PDX) model highlighting tumor volume, tumor growth and final tumor volume for the treatment of AV3 with and without co-treatment with gemcitabine.

Recently, different *in silico* models have also been focusing on the prediction of the therapeutic response to different TME targeting therapeutics, including the TME in PDAC [373,374]. For instance, Chen *et al.* simulated the response of PDAC towards tumor stroma targeting using PEGylated PEGPH20, a drug that breaks down hyaluronic acid, in combination with gemcitabine [375]. They observed similar results in their prediction model when compared to clinical data demonstrating how computational models can aid in drug development and evaluation for PDAC. Additionally, *in silico* models can also be used to analyze large amounts of data obtained from patient samples, but also from *in vitro* models such as OOC platforms, which eventually render the analysis of such data less time-consuming, more cost-effective and less prone to personal error [45,376,377]. The use of computational models can be a great addition to the conventional drug evaluation pipeline and eventually render it more efficient.

In summary, *in vitro*, *in vivo* and *in silico* models have all experienced impressive developments in recent years that render all models suitable at some stage of the development of therapeutics against PDAC. In particular, the focus on all models to include the PDAC typical TME components and characteristics is a crucial step towards *in vivo*-like models that are able to predict the response of patients towards new therapeutics and help to bring such products to the clinics. Furthermore, the use of such models can help in the

identification of novel mechanisms within the TME, which might eventually result in the identification of new therapeutic targets and could lead to promising and interesting novel treatment strategies against PDAC. However, it is crucial to highlight that there is no "one model fits all". A model, regardless of being *in vitro* or *in vivo* should always be selected based on the analysis criteria and the final aim of the study. The combination of the different individual assays, *in vitro* and *in vivo* models, will in the end lead to the most effective drug development pipeline. In summary, both *in vitro* and *in vivo* models have their unique advantages and disadvantages but both form a crucial part in the development of novel therapeutics for PDAC. The potential use of *in silico* models to further aid in drug development can render the process even more efficient which might eventually reduce the amount of time a drug needs to reach the market, which in the end is most beneficial for the patients suffering from PDAC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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